Biotechnology in Agricultural Chemistry

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Biotechnology in Agricultural Chemistry

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Foreword

The ACS SYMPOSIUM SERIES was founded in 1974 to provide a medium for publishing symposia quickly in book form. The format of the Series parallels that of the continuing ADVANCES IN CHEMISTRY SERIES except that, in order to save time, the papers are not typeset but are reproduced as they are submitted by the authors in camera-ready form. Papers are reviewed under the supervision of the Editors with the assistance of the Series Advisory Board and are selected to maintain the integrity of the symposia; however, verbatim reproductions of previously published papers are not accepted. Both reviews and reports of research are acceptable, because symposia may embrace both types of presentation.

Preface

BIOTECHNOLOGY AND ITS APPLICATIONS to agricultural chemistry are developing at a phenomenal rate. Since 1983, various symposia, including the one from which this book was developed, have been held to examine the various technical and applied avenues of research in this burgeoning field of science.

Although an abundance of literature has been published on biotechnology and recombinant DNA, no symposium has been published dealing with the applications of biotechnology to agricultural chemistry. In addition, other publications on the subject have not integrated biotechnology research with regulatory concerns about this research. This volume deals with state-of-the-art techniques in biotechnology and integrates this research with a perspective on regulatory action associated with biotechnology related to agriculture.

While organizing the symposium from which this book was developed, we talked with many people, both American Chemical Society (ACS) members and nonmembers, before and after making selections of topics and speakers. We tried to provide a balance and a wide range of biotechnology research areas related to agricultural chemistry. We have not provided an intensive, exhaustive, or detailed update on any aspect of the subject. Originally, we considered omitting the legal, social, regulatory, and ethical aspects, and including only the scientific or methodology issues. However, in this time and place we simply cannot congratulate ourselves and take pride in our scientific discoveries and marvelous inventions and ignore their possible adverse or long-term consequences.

The only place biotechnology can be sold as a science is on the stock market. Everywhere else, biotechnology is merely a means of developing tools or products that must be applied in some practical way. Biotechnology must compete with the performance, ease of use, effectiveness, and economy of all other methods for doing the same job. It must face the traditions and biases of the user. We want this book to not only instruct on advances in biotechnology, but also to open doors of communication and interactions between chemists, genetic engineers, regulatory agencies, environmentalists, and professionals in other disciplines.

This book is divided into four sections, each dealing with topics related to agricultural chemicals, pest control, and crop production. The first section deals with some of the recent developments in plant cell and tissue culture. The diversity of methodology presented in this section reflects the quickly evolving state-of-the-art areas of biotechnology and underlines the

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axiom that tissue culture is certainly one of the basic foundations for building agricultural chemical technology through recombinant DNA techniques.

The second section of this book deals with genetic engineering and selection techniques related to the development of biotechnology-based agriculture. The wide range of topics, from the development of single-celled photosynthetic organisms as a source of herbicide-resistant genes to rapid detection of *Salmonella* in food cultures, illustrates the versatility of biotechnology in the field of agricultural chemical research. The advent of microcomputer technology is certainly timely for the field of biotechnology, and the combination of both technologies creates a powerful thrust and view into what future research may hold for this field.

The third section of this book addresses microbial and other applications of biotechnology for agricultural chemistry. Use of microorganisms and microbial systems in degradation of waste pesticides and environmental hazards offers tools that until the present have not been available. Remedial environmental hazard cleanup is very costly. Use of microorganisms or enzymes that detoxify unwanted chemicals holds much promise in this area. In a report from a biotechnology study group formed to assist the Environmental Protection Agency (EPA), Martin Alexander and committee members suggested that the techniques of modern genetics and environmental microbiology can aid substantially in reducing the concentration or totally destroying chemical pollutants in surface and groundwaters, industrial and municipal waste treatment systems, and possibly in other circumstances. As the committee concluded, "Microorganisms have the advantage of providing low-cost, simple, and often highly effective means for chemical destruction."

Section three also contains a discussion of the use of genetically engineered microorganisms to produce agricultural chemicals. This area of research will certainly grow enormously in the future as scaled-up techniques of chemical production become cost-effective.

The fourth section of the book deals with economic, legal, safety evaluation, and regulatory issues for biotechnology related to agriculture. Various areas are covered, including laws governing applications of biotechnology patents related to agricultural products as well as regulatory and safety issues for biotechnology. Safety issues for biotechnology are of prime importance and concern in a field so young. Looking at perspectives, we see that safety issues of concern occurred late in the development of other agricultural products such as pesticides. A legitimate request is that past mistakes should not be repeated and that our society learn from the past how to produce both timely and safe products of biotechnology.

Several chapters in this book address state-of-the-art biotechnical safety and regulatory science as of mid-1985 and present the reader with an accurate historical perspective. At the time of the symposium from which

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this book was developed, the regulatory situation on biotechnology was dynamic and evolving rapidly, but has since become more solidified. The Biotechnology Science Coordinating Committee (BSCC) was established to serve as a coordinating forum for addressing scientific problems, sharing information and developing consensus, promoting consistency in the development of federal review procedures and assessments, facilitating cooperation among federal agencies, and identifying data gaps in scientific knowledge. Membership in the committee is composed of senior policy officials from the U.S. Department of Agriculture (USDA), EPA, Food and Drug Administration (FDA), and the National Science Foundation (NSF). The committee reports to the Federal Coordinating Council for Science, Engineering, and Technology (FCCSET). In addition, a domestic policy council was established at the cabinet level and contains a domestic policy working group on biotechnology composed of assistant administration-level personnel. On May 14, 1986, a Biotechnology Science Advisory Committee (BSAC) was established to provide expert scientific advice to the administrator at EPA concerning issues relating to risks and other effects of modern biotechnology applications.

Pieces of legislation pertinent to biotechnology include the Biosafety Act of 1985 and the Biotechnology Science Coordination Act of 1986. Each piece of legislation attempts to address the need for regulations and research to evaluate the risk of biotechnical products. Both bills were tabled at the time of this writing.

On an international scale, the Organization for Economic Cooperation and Development (OECD) has taken several initiatives in biotechnology. These initiatives include the formation of (1) an ad hoc group on safety and regulations in biotechnology, (2) the Directorate for Science, Technology, and Industry Committees for Scientific and Technological Policy, and (3) the Environment Committee to address safety of biotechnology materials.

Several applications for research and use of biotechnical products related to agriculture have been presented to EPA. To date, very few field studies have been performed. Of course, the ice-minus bacteria *Pseudomonas syringae* has been in the forefront of regulatory issues. The interjection of this issue into the judicial process and subsequent rulings represent a new approach by critics of biotechnology in that regulatory decisions appear to be made outside the executive branch of government.

We thank the ACS and our three sponsoring divisions—Agrochemicals (formerly Pesticide Chemistry), Agricultural and Food Chemistry, and Fertilizer and Soil Chemistry—for working with us in organizing the symposium from which this book was developed. We thank the contributors who gave so generously of their time and experience and who made this publication a valuable tool for scientists in the field of agricultural chemistry. We also thank our symposium chairpersons, Michael J. Haas

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Introduction

THE PUBLIC POLICY DEBATE on biotechnology development has shifted its focus from laboratory to deliberate-release issues. Thus, the feasibility and pace of agricultural applications is significantly influenced by newly formulated regulatory schemes, court proceedings, legislative deliberations, and evolving public perceptions.

Some observations on the factors relating to the biotechnology climates are necessary for understanding. Climates are the major areas such as governmental affairs, public acceptance, and overall business conditions that tend to affect the way an industry can develop apart from the scientific and marketing developments, which, of course, are the heart of biotechnology's progress to date. Each of these climatic areas is important and should be elaborated on.

Present Overall Picture of Commercial Biotechnology

What is the overall picture of commercial biotechnology? Many companies are pursuing a few initial products. The health care area is still in its infancy, although a burst of market activity is expected. Agricultural biotechnology is still at an earlier stage of development, and public issues are now focusing on field testing as an area of controversy. Industry has positioned itself to participate in formulating review regimes with a number of agencies that will be regulating biotechnology as products come to the marketplace. This activity is the quiet, day by day, two steps forward and one step back kind that is rather undramatic but makes the major difference as commercial entities prepare to show some results. Some marketplace results of significance are expected soon.

The major challenge for industry is to remain active in the regulatory dialogue that still requires resolution. But even more important is to look for creative opportunities to avoid government roadblocks. Over the past many years, industry has been reasonably traditional on numerous business issues, has taken predictable stances, and has eventually been pushed by the agencies or by the courts into making concessions. Biotechnology leaders have tried to get companies to see that industry might short-circuit some of this playing around for the benefit of getting early products out in the marketplace and for the benefit of gaining the public acceptance and enthusiasm for biotechnology that is lacking with respect to some other traditional industrial activities.

Industry, of course, would prefer to proceed cautiously rather than argue endlessly. That is, some legitimate doubts, questions, and uncertain-

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ties exist with respect to commercial biotechnology development. Biotechnology leaders have not pretended that these doubts do not exist, and industry has not taken the view that they know where they should be going and anything that gets in the way is merely inconvenient. Industry does take the view that some issues have to be considered by the public, and uncertainties have to be considered. Industry wants to participate in resolving these matters. Remember that the primary goal is to dispel the mystery of biotechnology—some of the falsehoods, some of the exaggerations, and some of the uncertainties—and the way to dispel these myths is to get the early products out in the marketplace where their benefits and dangers, if any, can be observed and evaluated by others.

Observations Concerning Climatic Areas

Public attention has largely shifted from biosafety issues to environmental protection issues. That is, with field testing of the new agricultural biotechnology imminent, are there problems with respect to the environment that will not adequately be taken care of? Biological warfare issues are also in people's minds through articles in some of the major papers and in other ways. These issues are going to continue to complicate people's understanding and their perceptions of commercial biotechnology. The public has difficulty understanding the various uses and issues of recombinant DNA. For the most part, this technology appears to be sufficiently complex and sufficiently not yet understood for little compartmentalization to occur. People do not think discretely about drug products or agricultural products. People think generally of biotechnology as being one big thing, and they either like it or dislike it for reasons that may or may not be particularly related to the product in question.

An example is the recent movie *Early Warning*, which is about the U. S. military conducting secret biological warfare experiments in Utah by using an agricultural company as a cover. In this movie, a bacterium or virus is created that turns people into homicidal maniacs when they are accidentally infected, and basically things get very much out of control.

This movie brought forth a couple of issues. First of all, an agricultural biotechnology company is used as a cover for germ warfare. This kind of movie is where people get the idea that the kind of work industry does is possibly related to troublesome activity. In the movie, the microbe turns people into murderous psychopaths. How plausible is this situation to people in the street? In agricultural biotechnology work, manipulating corn seeds or microorganisms is typical of what is currently being done. Would people believe that industry could accidentally or intentionally create, from these projects, a virus that could have the severe results of turning people into homicidal maniacs? I think that any particular scenario would seem sufficiently absurd as to be implausible, but also that people misunderstand biotechnology enough to agree with scientific scenarios that, on the surface,

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seem totally unlikely. The issue here is the amount of education necessary for people not skilled in the technical knowledge to become comfortable with biotechnology's potential and limitations.

The movie portrays scientists as trying to dominate regulatory decision makers. This portrayal reflects the fact that in our society today, a growing skepticism has arisen regarding scientists and the ways in which they ought to be permitted to either influence or participate in public policy decisions. So, as frivolous as the movie is, it carries a few valuable lessons.

Lessons can be learned when it comes to public perceptions. First, industry must realize that people like ourselves should try to participate in all biotechnology public policy discussions without regard to whether the particular issue at hand seems to be more appropriate for health care companies, or whether the issues are human genetic engineering, gene therapy, agricultural biotechnology, or otherwise. The point is that public attitudes are being formed right now and are being formed in ways that are going to affect everyone considerably. People involved in the biotechnology industry should try to participate in as many areas that affect public attitudes as possible. A second point is that many people will participate in issues in which industry has greater expertise, and so, particularly with respect to agricultural issues, industry should try to correct any misperceptions or views that are brought forth.

Education of the Public on Biotechnology

Education of the public on biotechnology is everyone's business. The biotechnology industry, despite its considerable strengths and resources, is not able to do the educating alone. People who have other perspectives aside from industrial perspectives can really aid substantially in communities by trying to make biotechnology a little bit more rational and by removing some of the myths.

Confidentiality Issues

The confidentiality question is an issue of current importance. The confidentiality issue is that as companies develop, various proprietary information is kept secret. This secrecy is the ordinary way that industry protects itself; and government, for the most part, adjusts to this secrecy in ways that are constructive. In biotechnology today, an increase in discussion of options has occurred in this area. Obviously, biotechnology is very competitive. Many companies strive initially to manufacture and market relatively few similar products; so for the product companies themselves, particularly the new biotechnology companies, their whole corporate viability could depend on the protection of certain proprietary information that gives these companies a competitive edge over others close behind.

With respect to the field testing stage, a further complication is that the

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10-acre threshold for experimental use permit data requirements is no longer observed. Therefore, a greater degree of information is provided today compared with earlier forms of testing. Business information is now collected by federal agencies at a much earlier stage than previously had been considered appropriate. The disclosure of this information could be very compromising for many small companies.

Because of the amount of public distrust of institutions generally, and industry in particular, industry needs to begin to examine the proprietary questions with a good deal of determination. Lines need to be drawn, if necessary, to formulate compromises; the government should not give away too much and industry cannot afford to let it give away too little. This task is not easy because the balance may be different with respect to individual products and may be hard to arrive at because of individual corporate philosophies. These questions of balance are extraordinarily important because they affect both the public's and the government's ability to monitor biotechnology. Industry can do better than the traditional approach of simply making concessions whenever battles are lost in the agencies and in the courts, or in the court of public opinion. A more practical approach is to participate actively and to get in the forefront of resolving this issue.

Government Climate

Biotechnology is currently beset by a wave of government activity as the transition from laboratory to marketplace begins to gather steam. U. S. public policy makers are generally quick to recite that our biotechnology leadership is a national asset of great value that we would be foolish to let dissipate. The translation of principles into specific policies applied to this novel and controversial field bridging several major industries is already complex and intriguing. A few of today's key uncertainties and their implications illustrate this point.

For example, an initial and comprehensive federal regulatory scheme is being rapidly formulated. Commercial applications will fall under the formal jurisdiction of the major federal regulators: the Environmental Protection Agency (EPA), the Food and Drug Administration, and the Department of Agriculture. Improvements sought by industry to the proposed regimes include greater jurisdictional clarity (e.g., give special attention only to subjects that are functionally novel), modified data requirements that would be more specific for scientific risk assessment purposes, and a demand for effective protection of collected proprietary data.

Probably of paramount importance, however, will be industry's attempt to secure at the outset a commitment for periodic review of the early regulations and a modification of restraints proven unduly conservative for public and environmental protection needs. This approach attempts to borrow from recent history, when the biotechnology public policy debate

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focused not on applications in the environment, as it does today, but on early laboratory research. When scientists in the mid-1970s publicly expressed concerns about conceivable biological hazards from certain recombinant DNA experimentation, an outcry arose from activists and critics who predicted epidemics and catastrophes if the technology was allowed to continue. A decade later, with an enhanced base of knowledge and experience, those early fears were found to be highly unrealistic. No one has been shown to have suffered as much as a sniffle from a great deal of laboratory and industrial scale-up work on recombined organisms. Whether the initial restrictions were a prudent response to a period of uncertainty or simply an uncritical overreaction is hotly debated. Technology development, however, was not stymied. The governing system of the National Institutes of Health (NIH) for approving experiments was sufficiently flexible that the restrictions initially compelled by both scientific uncertainty and political moods were relaxed. Today, the safety of closed-cycle (i.e., contained in a laboratory or in an industrial plant) biotechnology applications is not much in dispute, and an array of eagerly anticipated pharmaceutical products has resulted.

Now, as the first environmental applications of biotechnology prepare for field testing, varying degrees of confidence of avoiding negative impacts are again being aired. Critics argue that because of the unforeseen effects of pest organisms of the past (e.g., the gypsy moth or the kudzu weed), similar accidents will occur as biotechnological uses grow. Critics also feel that any new industrial activity that promises great benefits yet has not demonstrated commensurate dangers will probably do so in time. Proponents, on the other hand, argue that (1) genetic modification has no intrinsic danger, and (2) the problem organisms of the past have little relevance to whatever difficulties might be caused by genetic engineering because no genetic changes were involved; past problems arose when already successful organisms were introduced into a new environment lacking suitable controls. Also, proponents reject the pessimistic view of industrial development that something major always goes wrong. Proponents believe that cautious movement under government monitoring with organisms that have been observed and are well understood can lead to benefits without serious adverse effects. More difficult or questionable experiments can be done later, building on the feedback from initial ones. It is neither necessary for safety nor wise for public policy to be paralyzed by what appears to be little more than fear of the unknown. As the debate plays on, as new expressions of hypothetical dangers and catastrophes and a paucity of experience combine, an obvious question arises: Will the fact that recombinant DNA laboratory experiments, once assumed highly risky, mean that gene splicing research in other areas must overcome a presumption of danger and start with substantial restrictions?

Logic may not compel this result, but fears are hard to dissipate and

major unknowns are involved. Thus, business judgment argues for consenting to some initial restrictions that may later prove to be unnecessary. As experience from the early field testing accumulates along with other scientific evidence, industry's early concessions should be assessed, and if appropriate, adjusted. The capability for flexible and rapid response as circumstances warrant will be vital to ensure that necessary safeguards do not become burdensome restraints that impair U. S. competitiveness as commercial biotechnology matures worldwide. Whether the formal regulatory agencies can agree on the need for a flexible approach like that successfully adopted by the quasiregulator, NIH, is a critical question that biotechnology observers will watch closely.

Next consider the courts. Early this year, the U. S. Court of Appeals for the District of Columbia affirmed a lower court decision that kept NIH from approving a field test of recombined bacteria to be sprayed on plants to inhibit frost formation. The bacteria to be produced were not novel and were already isolated in the environment in small quantities. From an environmental protection standpoint, most scientists regarded the scaled-down experiment approved by NIH's Recombinant Advisory Committee as innocuous and looked forward to evaluating obtained field data.

The court ruled that NIH failed to take a hard enough look at possible adverse effects and ordered a more formal review, or an environmental assessment. This review was completed and, not surprisingly, anticipates no significant ill effects if the test is carried out. If the opponents press their case as expected, the lower court will next rule on whether the environmental assessment's conclusions were made after a hard enough look. If not, an even more formal environmental impact statement will be required. This environmental impact statement would take a minimum of many more months, if not years, to complete.

In new technology areas, when substantial unknowns and an aura of potential danger exists, reviewing courts could easily downplay any environmentally relevant information an agency considered. The reviewing courts could weigh more heavily whatever alleged deficiencies a skilled advocate could raise. Because judges are people too, their legal interpretations are likely to be influenced by their own perceptions of how credible or trivial hypothetical dangers may be. The jurists involved in the court proceedings mentioned considered biotechnology's alleged risks to be both plausible and troublesome. An overreaction is thus built into the system. Any new technology whose unknowns arouse fears can be subjected to years of legal sparring over how thorough industry's assessment was, while relatively innocuous experiments that would themselves contribute to the knowledge base are delayed. Thus, years may be required before the combination of experience and rising public acceptance diminishes the role of the federal courts as a potentially disruptive factor in some biotechnology planning.

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The decisions rendered to date do not apply to just industrial field testing. Still, the phenomenon is peculiarly American. European environmental law does not contain the unique procedural rigidities that could stifle innovation, and European law is far less inclined than ours to allow industrial innovation to be stymied by speculation alone. Commenting on an earlier stage of the litigation, the *London Economist* noted that although biotechnology had risks that should be regulated, "the regulation should be kept as flexible and informal as it can be. The danger, especially in America, is that it will instead be made legalistic and bureaucratized." Recently, several European countries have been aggressively courting U. S. biotechnology companies to build facilities abroad, and a few such agreements have already been reached. The uncertainty over U. S. courts' impact on early environmental applications makes foreign pitches an easier sell.

The principal result of the court activity has been to increase industry's realization that effective but acceptable environmental oversight would more likely be achieved by relying on the EPA, which is largely insulated from the legal procedural rigidities involved in the frost case, and not NIH. (Although the Department of Agriculture's review is, like NIH's, susceptible to the same legal excesses, modified plants generally arouse less concern than microorganisms.) For public acceptance reasons, this shift in industrial attitudes probably came earlier than it would have come otherwise and may prove quite constructive. Although NIH has done an admirable job of balancing innovation and safety considerations, its responsibilities are essentially promotional rather than regulatory. Thus, the NIH is subject to inherent criticism when functioning as a quasiregulator. Public confidence can be better maintained by shifting to a formal and rigorous regulator as industry moves away from research and more into production.

In a 1984 comprehensive report on biotechnology international competitiveness, the Office of Technology Assessment concluded that the United States was the world commercial biotechnology leader, but could have difficulty maintaining its position against determined foreign competitors. The summary stated, "If U. S. government funding for basic life science research continues its decline, the science base, which is the source of innovation in biotechnology, as well as in other fields, may be eroded." Earlier this year the administration announced, as part of an effort to reduce the federal deficit, that federal support would be reduced for biomedical research in 1985 below levels previously approved by Congress. NIH would be required to reduce the number of competitive grants awarded in 1985 by 23%, from 6500 to 5000.

As industrial competitors at a given point in time, different nations have inherent strengths as well as weaknesses. U. S. labor costs may stay high, and the degree of industrial cooperation may remain low compared with major competitors, but the elegance of our basic research in the life

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sciences is admired throughout the world. In biotechnology as in some other industrial areas, a few substantial strengths, if maintained and enhanced, may successfully compensate for our competitive deficiencies. Among determined competitors that have their own substantial capabilities, however, the future of a competitor who becomes complacent about his strengths is surely clouded.

Although today's uncertainties are not trivial, favorable resolution of government actions that bear on commercial biotechnology's development is possible. Regulatory attitudes can be adjusted, legal excesses can be modified by new judicial insight or by legislation, and developmental support waxes as well as wanes. Commercial biotechnology in the United States does possess considerable strengths, and its benefits to people worldwide are right for the times. Industry is looking for government to do more to support commercial biotechnology's staggering potential. Industry seeks an early-developed regulatory regime that is stable and allows for ready change as experience dictates; a system that allows industry to proceed cautiously yet without roadblocks; greater sensitivity to the need for positive support in such areas as exports, basic research, and judicial restraint; and an acknowledgment that government neutrality may be an inadequate industrial policy for the coming years. Our competitive position in commercial biotechnology today is uncommon, and pressures are building to draw out greater government support than is traditional in our system. A higher degree of government-industrial harmony may lie ahead.

Conclusion

Commercial biotechnology today exhibits many strengths, but the near-term developmental prospects are fragile. Our success will depend on the sustained efforts of many dedicated people of diverse backgrounds who will address themselves to the resolution of climatic as well as scientific and marketing issues.

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Chapter 1

Status and Overview of Biotechnology in Agricultural Chemistry

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Biotechnology has become the scientific buzzword of our age. One definition of "buzz" is "a confused murmur or flurry of activity." To a large extent this is a fitting explanation of what is going on under the diverse umbrella of biotechnology. No doubt the new techniques and tools, and the rapidly expanding knowledge of biology, genetics and biochemistry justify the enthusiasm, popularity, and expectations. Finally, mankind can experience the exuberance of having the power to "create life" with a new dimension. We are learning that this power still has limitations in spite of its great potential. Within this chapter are described some of the exciting scope and breadth of potential applications and products of biotechnology in the areas of crop and microbial production. This treatise is by no means complete or exhaustive, but it will hopefully provide a reasonable list to enlighten the layman and to motivate the expert. In addition, many of the basic tools and techniques used in biotechnology are outlined and described briefly. Some projections on the probabilities or possibilities of success, both short-term and long-term, as well as patent problems and some views on environmental and health concerns are also discussed.

Expectations and Limitations

Just prior to the symposium on "Applications of Biotechnology to Agricultural Chemistry" held at the ACS meeting in Chicago, September 9-12, 1985, John K. Crum, Executive Director of ACS, asked the pertinent question, "Does the American Chemical Society have a role in biotechnology?" The symposium and this subsequent publication serve as a strong confirmation that the future of biotechnology is not only compatible with, but dependent on chemistry as well as many other areas of science.

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The essential technological breakthrough that led to the current expectations and potential for biotechnology and, thereby, the birth of genetic engineering, took place in 1973 when Stanley Cohen of Stanford University and Herbert Boyer of the University of California at San Francisco found a simple way to combine DNA from two different organisms and then to clone identical copies of these recombined DNA molecules in bacteria.

During the past ten years, we have been inundated with claims as to how genetic engineering will revolutionize our lives. These revolutionary changes were to result from the development of new wonder drugs, novel varieties of crop plants and radically different methods of raising economically important farm animals. Recently, a prominent scientist gave an exaggerated notice that most of those involved in current agricultural chemistry, plant and animal breeding and other related technology will need to retire early or be retrained since there will be no need for pesticides and many other of our present tools (1). This statement is just as serious an exaggeration as those made by some scientists in the 1950's as the many spectacular herbicides were discovered, that we could now do away with the cultivator, hoe, and mechanical weed control. While it is true that the new tools created by biotechnology will replace some of our present methods, they will, in most cases, provide additional means of doing our jobs better.

For a wide variety of reasons, their introduction and impact will be a slow and gradual process. Reasonable predictions for its impact on world food production indicate that a 5 to 10 percent increase may result from biotechnology in the next quarter century, and that plant genetics might add 5 to 20 billion dollars a year to the value of major crops by the 1990's. The first genetically engineered crop plants will likely come on the market sometime between 1986 and 1990, with only a few products at first. By the year 2000, some predict that the new technology may dominate agriculture (1, 2, 3).

Plant scientists have achieved important and significant results in the past decade. However, the achievements to date in plant science do not begin to approach those of scientists cloning useful mammalian genes in various cells and microorganisms. The main reasons for this marked contrast are that the number of scientists interested in plant physiology and genetics are few, and the financial support for these areas of biotechnology is small, compared to the resources available in mammalian or microbial genetics. More importantly, the base line of knowledge available in plant genetics is much less than that available in microbial, or even animal cell genetics. Even if the plant genes coding for such important economic factors as drought tolerance, disease resistance, pesticide tolerance, or increased yield were characterized and identified, which is not yet the case, the ability to manipulate and transfer these genes at will to most plants is very limited. It can be stated with conviction that, in general, sufficient basic knowledge necessary to routinely and effectively engineer new and improved varieties of major crop plants via recombinant DNA does not exist today.

There is no doubt that the products of biotechnology and genetic engineering will have, if they haven't already had, a major impact on our current jobs and what many of us are doing. Any area of science or research tends to grow and then decline. Biotechnology is growing today as other disciplines are decreasing in importance or popularity. Although most of us will not need to be completely retrained, except by choice, we will certainly need to be flexible, willing to change and to develop new skills, and always keep learning. This book, hopefully, will serve as part of this process for some.

Like many fields of science, the ultimate benefit and application of our discoveries must often be found in other disciplines, or in interactions and combinations of disciplines. We must recognize that biotechnology, as chemistry, is a tool, a means to produce tools, or a means to an end, that serves in other fields. We should also appreciate that fame, fortune, influence and prestige are usually fragile; as easily and quickly lost as gained. Those of us who have lived through such cycles know that biotechnology will not always be the magic word and scientific attraction that it is today. That is not because it is overestimated or doesn't deserve the position and esteem it enjoys, but only that it is new and the realities, limitations and dependence upon other skills are not yet fully recognized. The attention of the public, politicians, and investors will soon be diverted to other priorities, long before the full impact and benefits of biotechnology have been discovered or developed.

If the possibilities inherent in biotechnology seem endless, man's trust in science is not, and we have seen many signs of rebellion. It is unfortunate that even now, in its infant stage, the products and early commercial applications of biotechnology are being threatened by skeptics who already fear that its risks will be greater than its benefits. As Dr. Robert Kaufman of Monsanto Company recently stated, "If it's going to cost $2.5 million every time we make a minor modification in a gene, then this field is dead" (4). Most of us would agree that the fears and objections of the critics are often unfounded or exaggerated, but few of us would disagree with their insistence that the burden of reasonable proof for environmental and health effects should be on those promoting their commercial use. Nor should we claim that exaggerations about biotechnology are the exclusive property of the non-scientists and uninformed critics. We have been exposed to about equal doses of science fiction both from the promoter of the merits and future potential of our science as from the hypochondriac who imagines that manmade monsters are lurking in the test tubes waiting to take over the world. One of the most ardent and vocal critics of biotechnology, Jeremy Rifkin, warns that its products will destroy mankind (5). In a more rational plea, Marc Lappé urging more social and ethical responsibility in the applications of biotechnology, stated "Recombinant DNA really is the millennium in biology, just as the discovery of nuclear fission was the millennium in physics. The power to do good or ill from both techniques is virtually limitless. We need not exaggerate our concern about what a few mad scientists may inadvertently create in a laboratory or

release into the environment, but we must hold accountable what whole industries or governments will choose to create with this newfound power" (6). It is quite possible that the glory years of biotechnology will be shorter than for most previous scientific disciplines. Containment or control after release, especially of engineered microorganisms, seems to be the greatest problem. Ability to properly monitor the products of biotechnology (e.g. microbes) and contingency plans in case of containment problems are also of concern.

While biotechnology has the potential to be a much needed and major industry for the American economy, it is facing a regulatory obstacle course that has frequently bogged it down in lengthy and labyrinthine approval processes. Even worse, once field tests or applications of biotech products have been approved, the decision has sometimes been threatened by, or fought out, in the courts. A supportive, reasonable, and coordinated regulatory environment, along with a continuing educational effort aimed at the public and government, must be established before biotechnology can achieve success, especially in the United States (7).

Potential Products of Biotechnology

It is not appropriate or possible to enumerate here a complete list of potential products or benefits from biotechnology. However, some of the major traits or products presently being developed or considered in engineered plants and microorganisms are listed in Table 1 and Table 2, respectively. While some genetic characteristics could be useful in both plants and microorganisms, and bacterial genes will often be used in engineering of crop plants, the products or traits are listed according to the most likely end use. Many of these traits are not discussed in detail in this publication. We have limited the scope of this book and symposium primarily to crop, soil and environmental applications, with very little attention given to animal and human uses of biotechnology.

Some of the effects of biotechnology on agricultural chemistry will be in direct competition with or in opposition to the use of chemicals, such as the development of pest resistant crops taking the place of present pesticides. Other applications will enhance or allow new uses for agricultural chemicals, such as: (1) the transfer of herbicide resistance into otherwise susceptible crops, (2) the use of genetically altered microorganisms to enhance the degradation of toxic or persistent chemicals in the environment, and (3) the use of engineered microorganisms in the synthesis and commercial production of specific isomers or chemicals for use in agriculture.

It has long been predicted that crop plants redesigned to be resistant to herbicides will be the first products of biotechnology of plants (8). There are a number of reasons why the genetic engineering for herbicide resistance is so attractive, including the following:

Table 1

Potential Plant Improvements and
Products From Biotechnology

1. Herbicide resistance in crops. This is one of the simplest, most popular, and probably will be among the first engineered traits to be of commercial application. It has also become a common development method and a model system from which to generate background experience in transforming plants. It could be one of the most profitable approaches for industries that can sell the herbicide-resistant crop seed as well as more herbicide. A wide range of crops resistant to many herbicides (e.g., triazines, glyphosate, paraquat, imidazolinones, sulfonylureas, etc.) is under development or in research. In the case of some of these efforts (e.g., atrazine-resistant soybeans), the objective may be more for the purpose of avoiding crop phytotoxicity or soil carry-over injury from previous applications than for direct use of the herbicide on the resistant crop. There are likely more than 100 companies or laboratories in the U.S. where biotechnology research is underway on herbicide resistance.
2. Seed or storage proteins altered or increased by the insertion of genes that alter the production of certain amino acids, enhancing the quality and nutritional value of seeds or grains. For example, much of our corn crop is fed to animals, but is deficient in protein containing essential amino acids, especially lysine. A number of high lysine mutants have been developed using classical genetic manipulation. The most notable is the opaque-2 variety of corn. More recently, high lysine rice has been developed. In the case of both rice and corn, the improvement in lysine content has led to significant losses in yield when the crop is grown under field conditions. In the case of high lysine corn, other agronomic difficulties have been encountered, such as harvesting, storage, and processing problems, due in large part to the softness of the corn kernel in the new variety. The premium price paid for the high lysine corn is not high enough to offset the 10 to 20 percent lower yield which the farmer obtains, and high lysine corn today is not a viable economic crop (15).

   The seeds of legumes and cereal grains provide humans with an estimated 70 percent of their dietary protein requirements, but the proteins in these seeds are also deficient in certain essential amino acids. Researchers have examined the possibility of genetically engineering the genes that code for these proteins to alter their amino acid composition, but they are complex, multigene families. Also, if we modify a single amino acid, it might affect the secondary and tertiary structure of the protein, which affects how it is folded and deposited as a storage protein (2).
3. Disease resistance in crop plants, such as engineering plants to turn on defensive mechanisms.

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4. Insect resistance in crop plants, by transferring genes that enable plants to make their own insecticides (BT toxin - see Table 2, item #3).
5. Drought resistance in crop plants.
6. Tolerance to flooding, excess moisture and poor soil aeration.
7. Tolerance to salt, salinity or toxic metals in crop plants, which would allow crops to be irrigated with sea or brackish water, or to be grown in vast areas not now suitable because of high alkali or salt content.
8. Tolerance to temperature extremes in crop plants.
9. Increase the cysteine and methionine amino acids (high-sulfur protein) in alfalfa. This is being attempted by Australian scientists by inserting pea genes (p-albumin-1) into alfalfa, anticipating enhanced wool production in sheep (16). The modification of leaf or forage proteins is probably more feasible than storage proteins, but there are likely sharp upper limits (2).
10. More efficient absorption and utilization of fertilizers. The increased uptake of phosphorous in early crop development has been promising.
11. Extending and increasing the ability of nitrogen fixation to major crops. This could greatly reduce our dependency on nitrogen fertilizers and the cost of crop production. It has been researched especially in cereal grains, rice and corn, but has not appeared promising due to the large number of genes involved (17), and the fact that biological nitrogen fixation consumes enormous amounts of energy and would have to be fueled, for the most part, by metabolism of carbohydrates from the plant itself (2, 15).
12. Increase the photosynthetic efficiency of crop plants. Much research has been done on the genes that code for the enzyme ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), which is probably the most abundant protein in the world and the key catalyst in photosynthesis. Rubisco consists of eight large protein subunits encoded by genes in chloroplasts and eight small subunits encoded by genes in the nucleus. Although no vector systems currently exist to alter genes in the chloroplasts, researchers are genetically engineering the genes that encode the small subunits. By increasing the efficiency with which Rubisco fixes atmospheric carbon dioxide, researchers hope to produce plants that will grow faster.
13. Development of shorter season varieties.
14. Modify growth stages or habits of crop plants for improved harvestability.
15. Improvement in taste or texture of vegetables and fruits, such as better tasting celery stalks and tomatoes with higher solids content (13). Somaclonal variations for selection of desirable variants, and somatic embryos (artificial seeds) have been especially useful in genetic engineering of improved vegetables. Several of these new crop varieties will be on the market within 2 to 4 years.

16. Increased crop yield or faster growth. These traits will be very difficult for the genetic engineer to manipulate directly since they probably involve many genes and interactions. It will be essential to determine how many genes and other complicating or limiting factors are involved in this and other traits before beginning a genetic engineering project.
17. New flowers, horticultural crops, and ornamental crops with improved traits, such as faster blooming, greater flowering, etc.
18. Develop crops that produce and excrete allelopathic chemicals or properties to better compete with weeds or insects.

Table 2

Potential Microbial and Other Improvements and Products From Biotechnology

1. Ice-nucleation active bacteria. This was the first microbial product of biotechnology for which approval of field tests was requested. This has resulted in a long court battle with no final conclusion to date. After 241 experiments, expenditures of $750,000, and five man-years of research, Advanced Genetic Sciences has not yet convinced regulators and the public that their modified Pseudomonas fluorescens (Frostban) is safe. The latest news on the similar ice-minus bacteria (P. syringae) being developed by Dr. Steven Lindow and the University of California, Berkeley, indicates that field tests with this microbe will also be delayed until 1987.
2. Improve the strains of nitrogen-fixing bacteria (Rhizobium) or the mechanism by which they fix nitrogen in plants. This has attracted new attention since it was found that many genes are responsible and a high energy loss takes place when plants (e.g. legumes) fix nitrogen, making it virtually impossible to transfer this trait in plants at the present time.
3. Develop a more toxic or cheaper strain of Bacillus thuringiensis (BT), or expand and improve delivery and application systems. Many companies are developing new technologies involving genetic manipulation of BT. Rohm and Haas and several other companies have inserted toxin-producing BT proteins into chromosomes of tobacco, attempting to provide protection against certain insects throughout the life cycle of the crop. Monsanto has implanted toxic BT protein in strains of Pseudomonas fluorescens, which they then use to coat crop seeds (e.g. corn) to protect the crop roots from insect (rootworm) attack. Mycogen uses a similar approach, but kills the bacteria by heat treatment before application (17).
4. Genetic engineering of other microbial and viral insecticides.

5. Development of microorganisms or production of enzymes for biodegradation of pesticides and other toxic wastes. This is a large and diverse potential that has long been of interest to microbiologists and others involved in handling and preventing environmental contamination from pesticide spills, wastes, container clean-up, plant effluents, on-farm spray rinsates, etc. Genetic engineering has greatly enhanced the interest and opportunities in these areas, while the recent awareness and long-term concern about groundwater contamination make it urgent and imperative that this technology be developed. It has been estimated that Superfund clean-ups will cost up to $100 billion over the next five years (18). Most of the clean-up will merely result in moving the problem from one place to another. The only totally satisfactory and permanent solution is to clean up the hazard in situ or where it occurs. Biotechnology offers the best and cheapest potential long-term for handling most of these problems (19).
6. Genetically engineered plant pathogens will provide additional tools to study pathogenicity (e.g. mutation analysis, stage of infection, host specificity and resistance, etc.), leading to improved crop resistance and disease control.
7. Use of monoclonal antibodies to detect and quantify specific pathogens, differences between virulent and non-virulent pathogens, and pathogen resistance to pesticides (fungicides), and to prevent disease by conferring passive immunity to an infectious agent.
8. Use of monoclonal antibodies and immunoassays for both field and laboratory determinations (qualitative and quantitative) of many environmentally and chemically stable pesticides, metabolites and other agricultural chemicals. Many companies and laboratories are actively developing and using this technology. While it will not completely replace GC, HPLC and other chemical assay methods for many analytical requirements, immunoassay methods are potentially much faster, easier and cheaper, once they are in place and operating, and they can be even more sensitive and specific (20). They can usually provide qualitative results within one to two minutes, and quantitative results within five minutes to a few hours, and will have a wide range of uses. They will detect pesticide in urine, blood, air, groundwater, food, plants and soil.
9. Development of mycoherbicides or site-specific beneficial plant pathogens.
10. Development of microbes that are able to protect crop plants from adverse environmental factors, such as soil salinity, acidity and toxic metals.
11. Development of microbial and enzymatic systems for production of natural product chemistry for agriculture, including pesticides, secondary metabolites (e.g. bialaphos, with herbicidal activity), optical isomers, specialty chemicals, etc.
12. Development of microbes for improved fermentation and utilization of agricultural biomass.
13. Use of plant cell culture systems to produce secondary metabolites or chemical substances.

1) It is relatively simple. The bioassay for the resistant trait can be fast, routine, and the resistant cells are easily separated from susceptible cells or plants.
2) Herbicide resistance or tolerance has occurred fairly readily in nature (especially to triazines) (8, 9).
3) Mechanisms of herbicide action and resistance have been well studied and they have often been dependent on a single gene.
4) Herbicide resistance in weeds can be useful, and has already been commercially transferred to crops by conventional breeding.
5) Considerably more money is spent on herbicides for weed control than for all other types of pesticides combined.
6) In spite of the great efforts and costs for weed control, it is estimated that weeds still cost more than $20 billion in lost crop production per year in the U.S. (9, 10).
7) The cost of genetically engineering a crop and extending the use of established herbicides is much cheaper than development costs for a new herbicide, assuming the technology is available and regulatory costs are not prohibitive.
8) Many of our best and most economical herbicides cause soil carryover, foliar injury, or other selectivity problems which can be remedied by developing resistant crops.
9) The basic research on herbicide resistance provides important new knowledge on plant physiology, biochemistry, and genetic engineering.
10) Herbicide resistance in crops offers flexibility in weed control methods which is very important, especially under conservation tillage and with minor crops.
11) Herbicide resistant crops could serve to extend the life of patents or expand use of herbicides in some chemical markets.

In order to find and manipulate the genes that code for resistance to herbicides, it is essential that we understand the mechanisms of action of herbicides in plant cells. It is fortunate that most herbicides interact with enzymes and other proteins in metabolic pathways specific to plants, such as amino acid synthesis and photosynthesis. Therefore, they are generally non-toxic to animals, and genetic engineering for these traits is specific for plants.

For example, glyphosate inhibits the enzyme, EPSP (5-enolpyruvylshikimate 3-phosphate) synthase, that catalyzes a step in the synthesis of the aromatic amino acids. Similarly, both the imidazolinones and sulfonylureas inhibit acetolactate synthase (ALS), the enzyme that catalyzes the first step in the formation of branched-chain amino acids (11). Triazine herbicides act by binding to a specific protein in the thylakoid membranes of the chloroplasts, preventing the flow of electrons and inhibiting photosynthesis (12).

Using different genetic engineering strategies, Monsanto and Calgene scientists have developed glyphosate resistant plants. Monsanto developed glyphosate resistant petunias, tobacco and tomatoes by engineering overproduction of EPSP synthase in these

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plants. Calgene has moved a gene for a mutant EPSP enzyme, which is not inhibited by glyphosate into tobacco, cotton, tomato and poplars. In addition, Molecular Genetics has developed corn plants resistant to American Cyanamid's imidazolinone herbicide, and DuPont researchers have identified mutant ALS genes, which confer sulfonylurea resistance, but they have not as yet reported genetically engineered resistant plants (2, 11, 13).

While it is difficult to predict which products and applications of biotechnology will succeed or be of most importance, it is certain that there will be both major and minor technological breakthroughs or discoveries that will have a great total impact on our agriculture and crop production of the future. We are just now seeing some of the early products of the pioneering efforts introduced for field trials or initial applications. Major breakthroughs are being made which will make it technologically possible to produce genetically engineered organisms earlier than many had predicted. However, the final and ultimate crops or organisms will likely require a longer time to develop or perfect than many biotechnologists expect.

Status of the Technology

One of the reasons for the great amount of time needed before major products of biotechnology will be ready for commercial use is the present limitations of biotechnology itself. For example, while scientists have a choice of ways to engineer new plants, a three-stage process is required, not all steps of which can readily be done with major crops, such as corn and soybeans.

The first stage in engineering a new crop plant is to establish a cell culture of the plant so that foreign DNA can be inserted. Tissue from any plant or seed part can be used to establish the initial cell culture. This donor tissue is put onto a synthetic growth medium, where it starts dividing and forms a type of wound tissue called callus. This callus is excised from the donor tissue and maintained in culture indefinitely by replenishing its medium. By manipulating the medium, particularly the hormone content, scientists can induce callus tissue to regenerate individual organs or a complete plant.

In the second stage, the desired genetic material or foreign DNA must be transferred into the cell in culture, and the cells that accept the new DNA must be separated from the remaining unchanged cells. There are several ways to insert new genetic material into plant cells once they have been established in the culture medium. The first and major method of DNA transfer is cell fusion. This can be accomplished several ways, all of which require using plant protoplasts, or plant cells whose cell walls have been digested away by enzymes. After the plant cell walls have been stripped off, the contents of adjacent cells can be forced to combine or fuse in a high-pH, high-calcium medium. Other methods of forcing cell fusion include polyethylene glycol treatment of plant membranes and a process called electroporation, in which pulses of electricity cause small pores

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to be created in the plant membranes, through which large molecules (e.g., DNA) enter. While healing, the protoplast membranes fuse together. This process, which has been used for a number of years on animal cell cultures, should work with plant cells. Another similar method that has had limited testing employs direct microinjection of DNA into the nucleus of protoplasts with partly regenerated cell walls. A fourth method for DNA transfer involves using synthetic liposome carriers to move the DNA. Liposomes are laboratory-made membrane packages into which DNA can be incorporated. A liposome can be fused with a plant protoplast by the same techniques used for cell fusion. However, none of the methods of transforming protoplasts can yet be useful for corn or cereal crops as no good system exists for regenerating plants from monocot protoplasts. A fifth and another frequently used as well as successful method requires using a vector such as a bacterium to carry the foreign DNA into the cell. The most prominent vector used in genetic engineering experiments has been the bacterium *Agrobacterium tumefasciens*, which causes crown gall disease in many dicot plants. This bacterium already has a mechanism for invading a plant cell and introducing its DNA into a plant nucleus. Genetic engineers insert the desired genetic information into the *Agrobacterium* DNA so that when the bacterium infects the cell, it carries along the new DNA. Unfortunately, this bacterium does not invade monocot crops, such as corn and cereals, so we must search for other suitable vectors that will infect monocot plants and carry the foreign DNA into them.

In the third stage, the transformed cells must be regenerated into whole plants, which must then be tested to ensure that the engineered trait has been successfully inherited and will be passed on to the next generation via the seed. There are two methods for getting cells that have accepted foreign DNA to regenerate. In one, called organogenesis, the cultured tissue produces a shoot apex, which is allowed to grow. After a certain point, the shoot is cut from the callus tissue and put into a different culture medium that allows rooting. When the shoot has rooted, it is put into soil and grows into a mature plant.

The other method of regeneration is somatic embryo formation, which can take advantage of somaclonal variations, or the inherent genetic diversity available in plant cells. Somaclonal variation refers to the observation that individual cultured plant cells do not always display the same genetic information (e.g., herbicide tolerance) as the plant from which the cells were derived. In addition, the ability to exploit somaclonal variation does not require the identification and transfer of individual genes. Somatic embryos are formed from nonreproductive cells. By manipulating the medium, researchers can often get the somatic embryos to grow into complete plants. However, these regenerated plants have often been sensitive to humidity, temperature changes, and fungal infections. When the somatic embryo has grown to maturity, seed is collected and checked to see if the desired trait has been inherited.

Even after the genetic engineer has successfully completed his work and has confirmed transfer of the genetic trait into a new crop, much more work is still ahead. Some of this, such as confirming that the genetic transfer is stable or compatible over many generations, that the trait is expressed only in the proper organ, at the proper time, and has no deleterious effects on the rest of the plant's genome expression or physiology, can be carried out by, or in cooperation with, the genetic engineer. However, much time, research, and testing will be required by the breeder, physiologist, agronomist and others after the biotechnologists or genetic engineers complete their job. Although the cooperation and interaction between biotechnology and these other disciplines have greatly improved in recent years, there is still a need for increased trust, collaboration, and joint research at many institutions. As stated by Hugh Bollinger of Native Plants, "Commercial prospects for new plant products may be inhibited by the difficulties of scaling up production and by the number of talented people trained in plant genetics" (13). Jaworski further emphasized that, "The most fundamental problem in applying genetic engineering to agriculture is a lack of basic biochemical knowledge about plants. We need to spend a lot more time -- and this is where I think we will see a great deal of activity in the next five to ten years -- on identifying agronomically important traits and the genes that regulate those traits. If we cannot do this, we are not going to be very successful in really making the agronomic improvements that we desire to make. We just don't have enough knowledge yet to understand how to regulate at will, and in a controlled fashion, the expression of a gene. There is a lot of basic research that has to be done in parallel with the applied research if we are going to be successful in moving the technology from the laboratory into the field" (2). The research needed to acquire this knowledge requires both greater cooperation between plant molecular biologists and traditional plant breeders and a commitment by the federal government to fund this kind of interdisciplinary effort.

Biotechnology Patents

The problems of patent protection, in addition to governmental regulations, add elements of risk to all genetic engineering projects. The commercial potential for products of biotechnology was made possible or at least greatly enhanced by two landmark decisions involving patents in recent years. The first was in 1980 when the Supreme Court decided that unicellular organisms could be patented (Diamond vs. Chakrabarty #447, U.S. 303). This new ruling went on to include plants, seeds and tissue cultures too, indicating that multi-celled, complex higher life forms with stems, fruits, flowers, etc., could also be patented. The second landmark decision was in October, 1985, very soon after the symposium, when the U.S. Patent Appeals Board awarded Molecular Genetics a patent for a genetically modified plant. This granted full patent protection to a corn plant, seeds and tissue culture

that Molecular Genetics scientists modified to overproduce the amino acid tryptophan, a characteristic that will make corn more nutritious for animals and may eliminate the need for dietary supplements. It could be the green light the biotechnology industry has been looking for to create new strains of plants, and certainly places patentability of engineered plants in a new light.

Prior to this action, the only way to obtain patents on plants propagated by seed was to do so on individual varieties through USDA. The Patent Office had previously insisted that it couldn't protect characteristics of plants. If Molecular Genetics had gone to the USDA to patent its characteristic, it would have had to file separate applications for each different kind of corn variety. Now covered by general patent law, genetic engineering for overproduction of tryptophan is protected in all corn plants. The standard for patentability of biotechnology products now seems to be that it only has to be novel, useful, and not obvious (14).

On the other hand, the process of applying for such protection is a thorny issue for agribusiness. Some of the difficulties include lack of available and knowledgeable manpower, lack of training within the patent agencies, and how to best prepare patents for optimum coverage. Most of the current examiners of new biotechnological applications have a background in chemistry. Frequently, they evaluate novelties in agribiotech in a manner different from that used by biologists. Besides, they can only spend about seven to eight hours with an application before they have to draft a judgment. Clearly presented applications which claim a narrow protection have the best chance of being approved. Broad claims may be rejected and carry the danger of being tied up in interference suits. These are costly and may last two to three years.

Nonetheless, biotechnology is here to stay and is a welcome blessing in providing new, better and faster means to develop crops, animals and food for our future and growing populations.

Practical Considerations in the Marketing and Applications of Biotechnology Products

The final factors necessary for a product of biotechnology or any science to be successful are economics, profitability and farmer acceptance. The user may not understand or care about the degree of scientific sophistication or what marvelous discoveries have led to the final product. He will only ask: "Is it simple to use, economical and will it make me a profit?" We must also realize that in most cases, the products of biotechnology will either be introduced in competition with chemicals or other products that are already accepted and are satisfying most needs, or they will enter new areas and markets where nothing is presently available or satisfactory. Both conditions present serious economic risks and acceptance problems.

With few exceptions, pest control in modern-day agriculture is based on chemicals. To successfully introduce a biologically-based pest management system into agriculture requires that a minimum of new technology or requirements be placed on the distribution system. This applies to all parameters of the product: shelf life, handling, compatibility with other pesticides, efficacy, and above all, cost per acre treated. A biologically based product must be able to fill a real need in the marketplace and must be able to do it through the established channels now used by chemicals.

Ultimate success will still be determined by how the plant or organism actually performs in the field. Genetically engineered crops can only succeed if they improve the farmer's balance sheet. Genetic engineering cannot alter basic rules of physiology. If improving the nutritional quality of a cereal results in substantial yield losses because of underlying basic processes, genetic engineering will not circumvent the problem. Genetic engineering potentially offers significant opportunities in crop agriculture, but only if basic economics are not adversely affected by the changes induced in the plant. Even then, a successful genetic engineering program may still require basic classical breeding technology to introduce the new trait into numerous varieties. Further, introducing a new variety into the marketplace requires that its characteristics be determined via field testing. Therefore, even if a substantially improved genetically engineered variety was available at the greenhouse level now, years of field testing would be required before widespread commercial utilization can occur (15).

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Chapter 2

Use of Plant Cell Cultures in Pesticide Metabolism Studies

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The use of plant cell cultures in pesticide metabolism studies has a history of only about 20 years, but pioneers of the technique have laid the groundwork for an increasing number of researchers interested in the advantages the cell culture systems offer. Most have chosen to use suspension cultures, because of the ease with which they can be manipulated, and the increased possibility for standardization of conditions from laboratory to laboratory. As an adjunct to whole plant studies cell cultures provide information as to the changes that structural modifications of a basic molecule may have on phytotoxicity, especially in detecting the inherent toxicity of a molecule that fails to penetrate or translocate in a whole plant. All of the comparative metabolism studies have shown that qualitatively there is little difference in pesticide metabolism in plants and cell cultures. Quantitative differences, either in the rate of conversion to a single product or relative importance of one pathway over another, do occur. Some of these differences may be of biological significance and would require the use of whole plants or plant parts to confirm the quantitative aspects of metabolism.

Historically, research concerning the fate and behavior of pesticides in plants has been conducted using the whole plant. Presently, investigators are also using highly purified cell-free extracts to study pesticide phytotoxicity and mechanisms of action. The gap between isolated enzyme systems and whole plants is being increasingly filled by studies utilizing algae (1, 2), mechanically or enzymatically isolated cells (3), and undifferentiated tissue or cell cultures (4). Cell cultures can be maintained for long periods of time and provide an axenic system that is easily manipulated. Additional advantages cited for the utilization of cell systems for metabolism studies include ease of extraction, possible elimination of purification steps, increased purity of the metabolite prepara-

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tions, use of lower levels of radioactivity, and the evaluation of metabolism dissociated from the complicating factors of uptake and translocation (4). Conversely, the technique cannot be used to evaluate the importance of pesticide metabolism by microorganisms that may be associated with the intact plant, nor can it be utilized to determine the importance of cuticular penetration or vascular transport on phytotoxicity. Asepsis is critical and requires specialized equipment to insure that contamination of the cultures does not terminate an experiment prematurely. Establishment and maintenance of cell cultures, especially suspension cultures, can become labor intensive. Although most plant tissue cultures may have the potential for photoautotrophic growth, they are not normally grown this way. Thus, they cannot be used to determine the toxicity of photosynthetic inhibitors, although secondary effects, including the influence of metabolism on phytotoxicity, may be estimated (5). Variability in the morphology, cytology, and physiology of cells may exist as a result of conditions employed in culture (4, 6) and must be recognized by researchers attempting to utilize plant cell cultures in pesticide metabolism studies.

Pioneers in the use of plant cell systems for studies of xenobiotic phytotoxicity and metabolism have laid the groundwork for an increasing number of researchers interested in the advantages the cell systems offer (4, 6-9). Greater emphasis on the identification of pesticide metabolites formed in whole plants and cell cultures has made evaluation of both systems more meaningful. Although the literature is nearly devoid of studies concerning characterization of enzyme systems from plant cell cultures that may be responsible for pesticide metabolism, reference to the cell cultures as a convenient source has been made (10).

Much of the literature on the use of plant cell cultures in pesticide research concerns herbicide metabolism, and it will be emphasized here. However, the comparative metabolism of other classes of pesticides in plants and plant cell cultures will also be reviewed. The advantages and disadvantages of plant cell cultures in pesticide metabolism studies will be presented, and a prognosis of the future attempted.

Establishment and Maintenance of Cultures

Callus Induction. Establishment of plant tissue cultures has been reviewed elsewhere (11) and a brief synopsis will suffice here. Induction of a callus is the first step in establishing a plant tissue culture. Callus is usually obtained from pieces of vegetative tissue free of microorganisms, but can also be established from intact seedlings grown from an aseptic seed, or from embryo explants. Vegetative tissue can be sterilized by immersion in 70% ethyl alcohol for 1 to 2 minutes, and/or by a 5 to 10 minute rinse in 20% commercial bleach (1% sodium hypochlorite). Although ethyl alcohol and sodium hypochlorite solutions are among the most frequently used surface-sterilants, there are a variety of other chemical agents available (11). The tissue pieces are triple-rinsed in sterile distilled water prior to placement on agar-solidified culture media. Aseptic conditions are essential for the long-term culture of plant tissue and cells, because the media used in the culture of plant cells and tissues may not promote the rapid growth

of contaminants. Therefore, a slow-growing organism can become widely distributed through cell transfers before the problem is detected. Rigorous asepsis must be adopted and cultures checked periodically for contamination.

Perhaps one of the reasons for the increased use of plant cell cultures in pesticide metabolism research is the ease with which the technique can be implemented as an adjunct to whole plant studies. Callus can be initiated from most plant species (11-13). Maintenance of sustained growth in culture is normally dependent upon the presence of growth-promoting substances in the medium; callus growth is not sustained in the presence of a sugar and mineral salts alone. A basal medium consisting of macro- and micronutrients and a carbon source (usually sucrose) may be amended with vitamins, amino acids, sugar alcohols, auxins and related plant growth regulators, cytokinins or synthetic substitutes, chelators such as ethylenediaminetetraacetic acid (EDTA), or complex natural extracts. Widely used plant tissue culture media include Murashige and Skoog (14), Gamborg (15), Nitsch and Nitsch (16), and Schenk and Hildebrandt (17).

Suspension Cultures. Although the culture of plant callus on agar-solidified medium is still the method *par excellence* for the routine maintenance of callus, limitations exist in the use of callus cultures for most metabolism studies. Gradations in gas exchange, growth response, and extrusion of toxic waste into the medium complicate the interpretation of experimental results. Standardization of experimental technique from laboratory to laboratory may also be more difficult to achieve when callus cultures are used (6). Therefore, most research on pesticide metabolism in plant cell cultures has utilized cell suspensions in which the callus is transferred to a shaken liquid media to form a suspension of dispersed cells. After the initial inoculation of the liquid medium with a callus, subsequent transfers are made by pipette to exclude large cell aggregates. Movement of the medium by shaking serves to aerate the culture and disperse the cells.

Estimation of Phytotoxicity. Several investigators have used cell cultures to study herbicide phytotoxicity (3, 5, 18-20). Callus and cell suspensions have potential in the estimation of phytotoxicity, especially in detecting the inherent phytotoxicity of a molecule that fails to penetrate into or translocate in a whole plant. Alteration of the molecule to enhance penetrability may provide a usable herbicide. Structure activity relationships have also been examined to determine the comparative potency of different plant growth regulators in cell cultures in comparison to whole plants (21).

Because cell cultures do not normally possess a fully functional photosystem, they fail to estimate the phytotoxicity of photosynthetic inhibitors unless secondary sites of action exist. The phytotoxicity and detoxification of the photosynthetic inhibitor metribuzin [4-amino-6-(1,1-dimethylethyl)-3-(methylthio)-1,2,4-triazin-5(4*H*)-one] was studied by Oswald et al. (5) in dark-grown cell suspensions of resistant and tolerant soybean cultivars. These researchers demonstrated that phytotoxicity was not restricted to photosynthesis. Enzymatic detoxification of metribuzin was inoperative in susceptible cell cultures and was suggested to be due to the

presence of a substance which inhibited the enzyme. Thus, although metribuzin is a photosynthetic inhibitor that might not be expected to inhibit the growth of dark-grown, achlorophyllous suspension cultures, phytotoxicity and detoxification of metribuzin in the cell cultures was independent of photosynthesis. In addition, the relative susceptibility of the soybean cultivars was reflected in measurements of the viability of the cell cultures. The photosynthetic inhibitors paraquat (1,1'-dimethyl-4,4'-bipyridinium ion) and diquat {6,7-dihydrodipyrido[1,2-α:2',1'-c]pyrazinediium ion} also inhibited the growth of non-photosynthetic callus-derived soybean [Glycine max (L.) Merr.] and peanut (Archis hypogeae L.) at concentrations as low as 1 uM (3).

Measurement of Growth. Several methods are available to estimate the growth and vitality of cell cultures. Fresh weight of calli and settled or packed cell volume of cell suspensions are commonly used measurements of growth (11). Less widely used growth parameters include cell number, dry weight, DNA and RNA content, and total protein. Fresh weight of calli can be determined without sacrificing the sample by weighing it under aseptic conditions and returning it to the agar-solidified medium. Similarly, the growth of cells in a suspension culture can be determined non-destructively by measurement of settled or packed cell volume. The culture is placed in an autoclaved, graduated, conical centrifuge tube and the cells allowed to settle for 20 minutes. Cells of some species will not settle well and should be centrifuged at 2000 g for 5 minutes (11). Measurement of optical density of cell suspensions following sonication has been utilized to measure growth in batch cultures (22) and appears to be a rapid, accurate means of growth assessment. Optical density measurements are also used to maintain the constant growth rate of continuous cell cultures grown in turbidostats (11, 23). Other rapid estimates of phytotoxicity have included precursor incorporation into macro-molecules, membrane permeability to fluorescein, and reduction of triphenyltetrazoliumchloride (24). Davis et al. (25) compared dry weight, fresh weight, packed cell volume, settled cell volume, spectrophotometric absorbance of sonicated aliquots, and electrical conductivity as methods for the estimation of xenobiotic phototoxicity in cell cultures. A summary of the comparison of those methods is presented in Table 1. The authors suggested that plant cell cultures could be used to estimate the effects of xenobiotics on growth and numerous biochemical parameters, but they could not be readily used as a rapid, reliable primary screen by themselves. Moreover, certain aspects of their study cast doubt on the applicability of the cell cultures for long-term studies of phytotoxicity. Because the cells have to be transferred frequently to remain growing, changes occur with time in the physical, and perhaps the physiological, characteristics of the cells, that may influence their tolerance of the chemical (25).

Dosage. The choice of pesticide dosage for use in metabolism studies conducted with plant cell cultures will vary with the plant species and the pesticide; however, it is important to establish the phytotoxic response for the conditions under which the metabolism portion of the study is run, since metabolism may be affected by the condition of the cells during treatment (4, 6, 27). In addition, it may

Table 1. A comparison of methods used to measure growth of soybean cell suspensions

Technique	Procedures	Comparison: Advantages	Comparison: Disadvantages
Oven-dry weight of entire flask content	Filter, wash, and oven-dry cells	Extremely simple. Data can be obtained readily with minimal training. Usually quite consistent and reliable. Requires normal laboratory equipment.	Entire sample destroyed. Requires considerable space if Erlenmeyer flasks are used. Requires two weighings per sample. Dry weight declines after reaching maximum.
Oven-dry weight of 3-mL aliquots	Same as above except can use same flask throughout if flask is large.	Same as above. Space is minimal. Can sample same flask if the flask is large.	Liquid volume decreases with sampling, changes environment of cultures. Variability often high. High risk of contamination if flasks are sampled frequently.
Absorbance of sonicated cells	Aliquot sonicated. A_{525} measured.	Results comparable to dry weight. Sample same flask if flask is large.	Sonication takes time, extra manipulations, and an extra piece of equipment. Standard deviations high. Sonication may not disrupt cells completely. A_{525} must be taken quickly and consistently.
Packed cell volume	Centrifuge aliquot in graduated tubes, read volumes.	Simple, direct, fairly rapid. Growth curves form a plateau without declining.	Variability often excessively high. Requires several manipulations (pipetting, centrifugation, opening of flasks). Sampling is difficult if suspension is dense.
pH	Use single probe glass electrode; aliquot or entire contents.	Simple, rapid, can use any volume desired as long as probe is covered adequately. Cell clumping or viscosity does not interfere.	pH changes not always indicative of growth pattern.
Electrical conductivity	Use conductivity cell; aliquot or entire contents.	Same as pH. This method is one of the best of those used in terms of simplicity, reproducibility, and correlation with dry weight changes.	Time of minimum conductivity ("maximum growth") frequently does not correspond exactly with time of maximum of dry weight changes. Extra equipment required. Some of the medium is removed during sampling, changing environment.
Settled cell volume	Use nephalometer flasks with side arm and cleanout port.	Extremely simple; easy to manipulate; can use same flask throughout without risk of contamination. Very good to establish marked inhibition of growth.	Occasionally growth may vary from dry weight measurements; precision may be low. Flasks with cleanout ports opposite side arm tubes are essential.

Source: Data are from reference 25.

be important to establish if the cell cultures mirror the response of the whole plant. Phytotoxicity measurements may also indirectly provide information concerning the ability of the cell cultures to metabolize the product to a non-toxic form. If two or more metabolic pathways are operative in the detoxification of pesticidal chemicals, quantitative differences in metabolism may also affect phytotoxicity.

Unfortunately, in some instances it is impractical to conduct phytotoxicity tests and metabolism experiments under the same conditions. Swisher and Weimer (26) conducted a study of comparative detoxification of chlorsulfuron in cell suspension cultures of leafy spurge and Canada thistle in an effort to correlate metabolism and phytotoxicity. Phytotoxicity tests utilized approximately 0.5 g of cell biomass treated with chlorsulfuron concentrations from 10^{-9} to 10^{-7} M. The volume of the cells was determined at weekly intervals for 3 weeks and growth inhibition calculated at the conclusion of this time period. Due to the low specific activity of the ^{14}C-chlorsulfuron (5.99 µCi/mmole), concentrations of 10^{-6} M were used in 3 day metabolism studies with 7 to 10 g cell biomass. Direct comparisons of phytotoxicity and metabolism could not be made, but the authors concluded that metabolism contributed to the differences in phytotoxicity observed between cell cultures of tolerant leafy spurge and Canada thistle cell cultures (Figures 1, 2). Differences in the metabolism of chlorsulfuron by these two species could be rapidly assessed by using a relatively large cell biomass. Phytoxicity tests were conducted over a longer period of time to allow the cell cultures to outgrow any initial inhibition that occurred before detoxification reduced chlorsulfuron concentrations below phytotoxic levels. Leafy spurge cells recovered, but Canada thistle did not. Presumably, metabolism of the pesticide to a non-phytotoxic product is the reason that some cell cultures begin to grow after 2 to 3 weeks in the presence of a concentration that initially appears to be phytotoxic (25).

Pesticide Metabolism in Plant Cell Cultures

Callus Cultures. Much of the published research on pesticide metabolism in plant callus cultures has been conducted in only a few laboratories. Much of the justification for the use of callus cultures, rather than the more uniform suspension cultures, is that the gradations in nutrient availability, toxic waste extrusion, and the relative nonhomogeneity of the culture may be used to better estimate whole plant metabolism, especially if the culture conditions are manipulated (4). In a series of papers, metabolism of 2,4-D in whole plants and callus tissue was systematically compared (27-31). Several of these studies utilized calli transferred from agar-solidified media to liquid medium at the time of treatment. Approximately 10 g of tissue was transferred, treated with 1-5 µCi of ^{14}C-2,4-D, and maintained with gentle shaking for the duration of the incubation period (27-29, 31). One study (30) utilized a direct injection technique in which 10 ul (5 µCi) of ^{14}C-2,4-D in ethanol:acetone was injected directly into the calli. Although solvent phytotoxicity has been reported (3), it was not noted by Feung et al. when direct injection of small amounts of solvent were used.

Qualitative and quantitative differences in metabolism were

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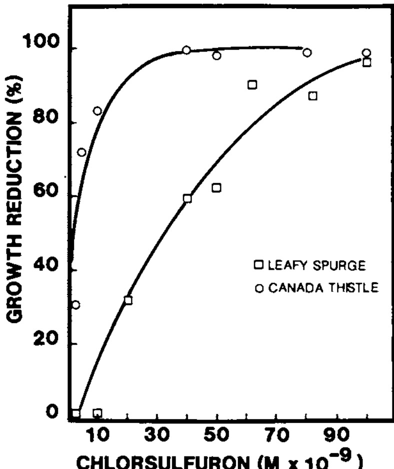

Figure 1. Effect of chlorsulfuron concentration on growth of leafy spurge and Canada thistle cell cultures 3 weeks after treatment. Each point represents the mean of four or more replications. The lines are fitted to the data by regression analysis. (Reproduced with permission from reference 26. Copyright 1986, Weed Science Society of America.)

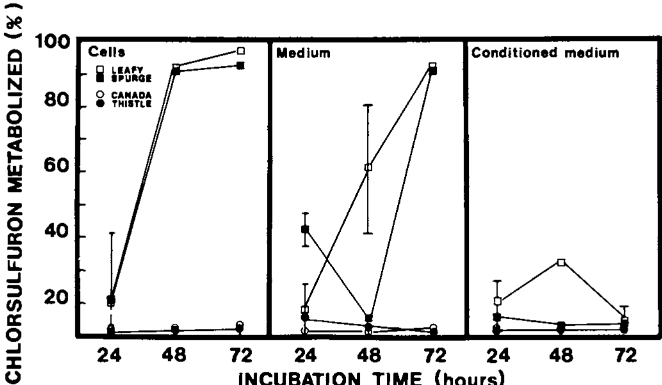

Figure 2. Metabolism of ^{14}C-chlorsulfuron in leafy spurge and Canada thistle cell cultures or in conditioned medium at 24, 48, and 72 h after application of 0.10-uCi ^{14}C-chlorsulfuron. Conditioned medium had been passed through a 20-um filter to remove cells at the time of treatment. Closed and open symbols represent separate experiments. Each data point is the mean of two replications. Vertical bars represent the standard error of the mean; when not present, the standard error was less than 1%. (Reproduced with permission from reference 26. Copyright 1986, Weed Science Society of America.)

observed in these experiments. Quantitative differences led to the development of a scheme of 2,4-D metabolism comprised of three major pathways - ring hydroxylation, amino acid conjugation, or sugar ester formation (30). Ring hydroxylation and amino acid conjugation were predominant in soybean callus and soybean plants, while sugar ester formation predominated in corn (30) and rice (33).

Quantitative differences in 2,4-D metabolism were also observed as a result of herbicide concentration and tissue age in soybean callus cultures (31). Unmetabolized 2,4-D constituted most of the ^{14}C extracted from three-week old callus tissue, and the concentration of free 2,4-D increased 32-fold in the tissue as external 2,4-D concentration was increased from 10^{-6} to 10^{-5} M (Figure 3). Aqueous (glycosides) and ether soluble metabolites (amino acid conjugates) were present in lower amounts, and increased slowly over the 10^{-6} to 10^{-5} M concentration range. In nine-week old callus cultures, ether soluble metabolites (2,4-D amino acid conjugates) were the major components of the tissue, and increased four-fold over a three-fold increase in 2,4-D concentration in the external medium (Figure 3). The concentration of 2,4-D remained low and relatively constant as it was converted to amino acid conjugates. Aqueous soluble metabolites formed a relatively minor component in nine-week old callus.

Correlation of metabolism with physiological status of the calli indicated an inverse relationship between the mitotic index and 2,4-D metabolism. The authors suggested that free 2,4-D may be required for cell division and expansion, and that 2,4-D conjugation may be regulated by endogenous levels of free amino acids, which were present at lower levels in younger callus (30). Support for this hypothesis was indirectly provided by Montague et al. (34) when they showed that embryogenic carrot cells contained more free 2,4-D than soybean callus cell suspensions. Conversely, amino acid conjugates comprised only 3% of the radioactivity recovered from embryogenic carrot cells 3 days after incubation with 1 ppm ^{14}C-2,4-D, compared to 89% and 80%, respectively, from 'Dare' and 'Wayne' soybean cultures.

Studies of 2,4,5-T [(2,4,5-trichlorophenoxy)acetic acid] metabolism in soybean callus tissue also showed that formation of amino acid conjugates was a major metabolic fate of the herbicide (35). No direct comparisons were made with soybean plants in that study, but others have reported decarboxylation (36), hydroxylation (37, 38), and glycoside formation of 4 OH-2,5-D (39) as minor metabolites in various plant species. Minor metabolites were detected, but not identified in soybean callus tissue (35).

Suspension Cultures. Despite valid reasons for the use of callus cultures in pesticide metabolism studies, most researchers have chosen to use suspension cultures, because of the ease with which they can be manipulated, and the increased possibility for standardization of conditions from laboratory to laboratory. All of the published literature concerning pesticide metabolism in plant cell suspensions has been performed utilizing cells grown in a closed system and a finite volume of nutrient medium. These batch cultures are characterized by the growth cycle shown in Figure 4 (11). Growth of the culture (measured by increase in cell number, protein, biomass, or other physiological parameters) proceeds after a lag period during which no growth can be measured. The exponential stage

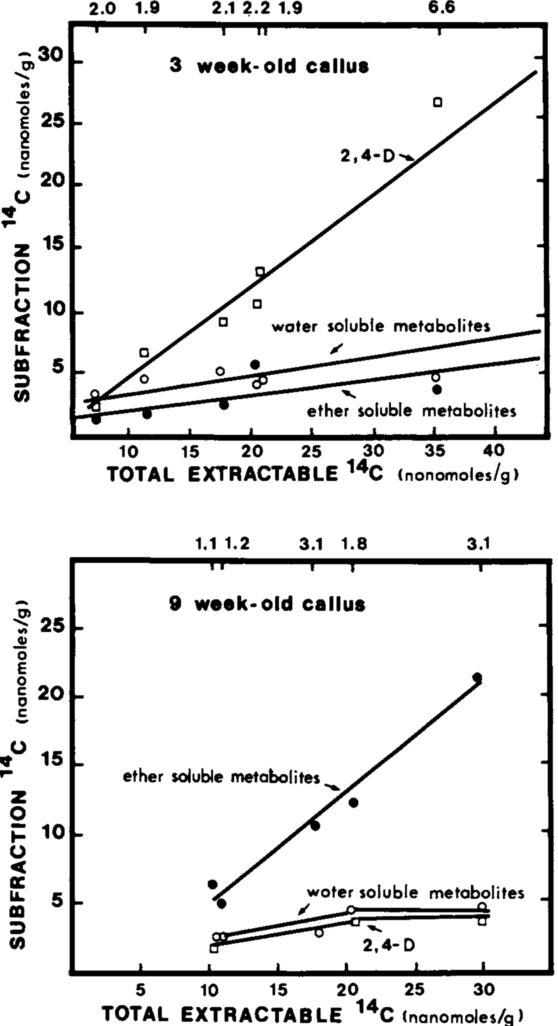

Figure 3. Concentration of 2,4-D or 2,4-D metabolites found in various subfractions vs. total concentration in the ethanol extract of 3-week-old soybean root callus tissue (top) or 9-week-old soybean root callus tissue (bottom) following incubation for 48 h with various levels of ^{14}C-2,4-D (1.8×10^{-6} to 1.1×10^{-5} M). The free 2,4-D was subtracted from the total ether-soluble metabolites (mostly amino acid conjugates). (Reproduced with permission from reference 31. Copyright 1978, American Society of Plant Physiologists.)

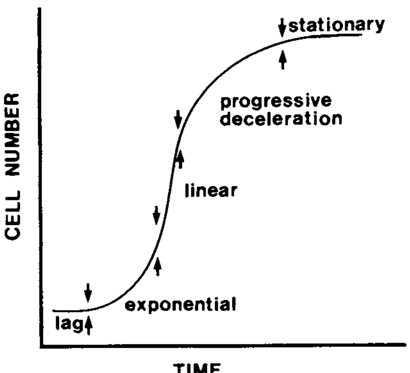

Figure 4. Growth curve for a batch cell suspension culture. (Reproduced with permission from reference 11. Copyright 1973, University of California Press.)

is a finite stage early in the growth cycle of a batch culture during which the specific growth rate (usually cells/ml culture) increases logrithmically with time. The specific growth rate then begins to decrease to become linear with time (linear phase) and then at a progressively faster rate (progressive deceleration), until no further growth can be detected. During each subsequent transfer, the cycle is repeated. Thus, the rate of growth of a batch culture changes almost continuously from one subculture to the next. Moreover, batch cell cultures are usually characterized by unbalanced growth in which one parameter changes at a different rate than another, contributing to a continually changing cell composition (11). Changes in metabolic and enzyme activity also occur during the growth of a batch culture, and have been shown to affect the results of pesticide metabolism studies (40, 41). In a comparison of propanil [N-(3,4-dichlorophenyl)propanamide] in rice plants and suspension cultures, Ray and Still (40) showed that propanil metabolism to 3,4-dichloroaniline and propionic acid by an aryl acylamidase was dependent upon the chronological age of the plants and the cell suspensions. When amidase activity was assayed in crude homogenates of rice root callus suspension, no activity was observed until five days after inoculation of the cells into the culture medium containing propanil. Comparison of growth curves and enzyme activity showed that the increase in amidase activity corresponded to the stationary phase of growth. In a similar study conducted with diphenamid (N,N-dimethy-α-phenyl benzeneacetamide), Davis et al. (41) compared the metabolism of the herbicide in soybean [Glycine max (L.) Merr.] cell cultures during early exponential stage, late exponential stage, and stationary stage of the growth cycle. Diphenamid was metabolized by cells of all ages, but was metabolized about two times more rapidly in early log-phase cultures as in stationary cells, based on unit mass and unit time. The absence of balanced growth in batch cell suspension cultures does not preclude their use in pesticide metabolism studies, but an accurate growth analysis prior to herbicide application, and an appreciation for the rapid changes that can occur during the growth cycle are essential.

Comparative Pesticide Metabolism in Plants and Plant Cell Cultures

Herbicides. In addition to the herbicides already discussed, comparative metabolism studies have been conducted with fluorodifen [4-nitrophenyl 2-nitro-4-(trifluoromethyl)phenyl ether] (42), diclofop-methyl {[methyl 2-[4-(2,4-dichlorophenoxy)phenoxy]propanoate} (43), pyrazon (chloridazon) [5-amino-4-chloro-2-phenyl-3(2H)-pyridazinone] (44), cisanalide (cis-2,5-dimethyl-1-pyrrolidinecarboxanilide) (45), MCPA [(4-chloro-2-methylphenoxy)acetic acid] (46), and mefluidide {N-[2,4-dimethyl-5-[[(trifluoromethyl)sulfonyl]amino]-phenyl]acetamide} (47). Most of these studies showed that metabolism as qualitatively similar; the same metabolites were isolated from whole plants and plant cell cultures, but quantitative differences in rates of conversion were noted (Table 2).

Insecticides. Comparative studies of insecticide metabolism in plants and plant cell cultures have included the organochlorines DDT [1,1,1-trichloro-2,2-bis-(4-chlorophenyl)-ethane] (48, 49), aldrin (1,2,3,4,10,10-hexachloro-1,4,4a,5,8,8a-hexahydro-exo-1,4-endo-5,8di-

In Biotechnology in Agricultural Chemistry; LeBaron, H., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

Table 2. Comparative pesticide metabolism by plants and cell cultures

Herbicide	Species	Results	Reference
fluorodifen	tobacco	similar; glucoside of 4-nitrophenol was major metabolite	42
diclofop-methyl	*T. aestivum* and *T. monococcum*	similar; phenolic conjugates of ring hydroxylated diclofop	43
diphenamid	soybean	quantitative differences; age of cell cultures was important in metabolism	32
chloridazon	sugarbeet poppy mung bean soybean tobacco parsley	No direct comparison with whole plants made. Metabolism was species dependent in cell cultures. Hydroxylation occurred in poppy and sugarbeet, but chloridazon was not metabolized in mung bean, soybean, tobacco, or parsely.	44

Table 2--Continued on next page

Table 2--Continued

Herbicide	Species	Results	Reference
Mefluidide	johnsongrass soybean	similar; sulfoxidation to sulfoxide and sulfone	47
propanil	rice	quantitative differences; age of cell cultures was important in metabolism	40
cisanilide	carrot cotton	quantitative differences; cell cultures had reduced capacity to form glucosides	45
2,4-D	soybean wheat corn rice carrot sunflower tobacco Jackbean	quantitative differences; based on culture age, explant source, variety, and chemical concentration. Cell cultures routinely used in 2,4-D metabolism studies	31,33,49
	wheat	similar; methyl hydroxylation was the major metabolic product in whole plants and cell cultures	46

Insecticide	Species	Results	Reference
DDT	parsley soybean wheat	similar, slow conversion to DDE, DDA, and hexose esters of DDA; unidentified polar metabolites formed in small amount	47,48
EL 494 (insect molt inhibitor)	soybean	similar hydrolytic products	53
carbaryl	tobacco	quantitative differences; cell cultures had reduced capacity to form glycosides of N-hydroxymethyl and ring hydroxylated carbaryl, but increased capacity to form the -naphthol glycoside	52
aldrin	bean potato	quantitative differences, explant source, and culture media influence the distribution of the metabolites	50

Table 2--Continued on next page

Table 2--Continued

Insecticide	Species	Results	Reference
dieldrin	bean potato	quantitative differences; same as aldrin	50
lindane	numerous	quantitative differences, rates of metabolism faster in cell culture systems	51
Fungicide			
carboxin	peanut	similar except that bound residues were greater in whole plants. Oxidation to the sulfoxide and hydrolysis of the carboxamide bond appeared to be the two primary reactions.	54
PCNB	peanut	quantitative differences, major differences in conversion to pentachloroaniline that were dependent upon the O tension of the culture	55

Unclassified Xenobiotics	Species	Results	Reference
PCPMS	cotton carrot tobacco	quantitative differences. Rates of sulfoxidation in cell cultures were greater than in whole plants	58
benzo α pyrene	parsley soybean	no direct comparisons made, but several metabolites were isolated from cell cultures. Benzo pyrene was also associated with the lignin fraction	56
PCP	soybean wheat	incorporation into insoluble metabolites in whole plants; incorporation of PCP into lignin fraction was demonstrated in cell cultures	57

methanonaphthalene) (50), dieldrin (hexachloro-epoxy-octahydroendo, exo-dimethanonaphthalene) (50), and lindane (gamma isomer of 1,2,3,4,5,6-hexachlorocylohexane) (51), as well as carbaryl (1-naphthyl N-methyl carbamate) (52), and the insect molt inhibitor EL-494 {N-[[[5-(4-bromo-phenyl(-6-methyl-2-pyrazinyl]amino]carbonyl]-2,6-dichlorobenzamide} (Table 2). The metabolism studies conducted with DDT are noteworthy because they demonstrated that this persistent chemical can be metabolized by plant cells to the polar metabolites DDA [2,2-bis-(4-chlorophenyl)acetic acid] and its methyl ester (48, 49). It remains to be seen if plants grown in a sterile environment can also metabolize DDT, but it raises the possibility that they may serve to moderate the bioaccumulation of DDT in the food chain by conversion to such products as DDA (48, 49).

In a very thorough study of carbaryl metabolism in tobacco suspension cultures, at least eight metabolites were identified, including a chloesterol derivative that the authors suggested may represent a new detoxification mechanism in plants (52). They also suggested that the tobacco cell line used in the study had certain advantages in studies of pesticide metabolism. The cell line grew well in the dark, so photochemical effects on the chemical could be eliminated. The tobacco cells proliferated rapidly on an inexpensive, fully defined medium. Additionally, the biochemistry of the cell line had been well investigated and could perhaps augment metabolism or mode of action studies with other chemicals.

Lindane metabolism has been studied in whole plants and cell cultures of numerous species (51). Metabolism of ^{36}Cl-lindane (0-6.8%) and release of ^{36}Cl from suspension cultures occurred during 12 to 28 day incubations, but little ^{36}Cl was released from intact plants.

Fungicides. Metabolism of the fungicides carboxin (5,6-dihydro-2-methyl-N-phenyl-1,4-oxathiin-3-carboxamide) and PCNB (pentachloronitrobenzene) have been studied in peanut cells and plants (54, 55). Carboxin sulfoxide was the most abundant metabolite in both peanut plants and cell suspension cultures. In addition, carboxin sulfone, the hexose ester of 5,6-dihydro-2-methyl-1,4-oxathiin-3-carboxylic acid, malonanilic acid and the β-D-O glucoside of p-hydroxymalonanilic acid were also recovered from peanut plants and cell cultures (Figure 5). The most obvious difference in the metabolism of ^{14}C-carboxin in peanuts and peanut cell suspensions was in the formation of bound residue (54). The greater lignification and production of secondary products may account for the increased incorporation of ^{14}C into bound residues in whole plants. The primary difference in metabolism of PCNB in peanut roots and cell suspensions was also in the formation of bound residues (55). In addition, the reductive conversion of PCNB to pentachloroaniline was dependent upon the oxygen tension of the cell culture. When maintained by shaking at conditions of normal oxygen tension, pentachloroaniline accounted for only 2% of the ^{14}C in peanut cell suspensions. However, when oxygen tension was reduced (by elimination of shaking) pentachloroaniline was increased to 10% of the applied dose.

Unclassified Xenobiotics. Although they do not represent specific pesticidal classes, other xenobiotics have been studied in plants and plant cell cultures. Among these are the carcinogen benzo[ ]pyrene

Figure 5. Metabolism of carboxin in peanut and peanut cell culture. Values in parentheses are the percent of the total recovered ^{14}C found in the illustrated form from peanut plant and peanut cell suspension cultures. (Reproduced from reference 10. Copyright 1984, American Chemical Society.)

(56), the chlorinate phenol PCP (pentachlorophenol) (57), and an aromatic alkyl-sulfide (58) (Table 2).

The examples above serve to illustrate certain generalities that can be drawn from comparative metabolism studies in whole plants and plant cell cultures. All of the comparisons have shown that qualitatively, there is little difference in pesticide metabolism in plants and cell cultures - the same compounds are isolated from each system. However, quantitative differences either in the rate of conversion to a single product, or relative importance of one metabolic pathway over another do occur. Some of these differences may be of biological significance and would require the use of whole plants or plant parts to confirm the quantitative aspects of metabolism.

Future Directions

Standardization of Method. Although the applicability of cell culture techniques to pesticide metabolism and toxicity studies has been established (4, 6), there are limitations to their use, and possibilities for standardization that may best tap the potential of this research tool.

The utilization of cell cultures as routine toxicity screens must be augmented by whole plant studies to establish that the cell cultures not grossly over or underestimate the phytotoxicity of a xenobiotic. As an adjunct to whole plant studies they provide information as to the changes that structural modifications of a basic molecule may have on phytotoxicity to the whole plant. Perhaps one of the most valuable purposes the cell cultures serve is to provide information as to whether or not cell cultures of different species grown under identical conditions can tolerate a specific xenobiotic (25).

Another prospect for the use of cell cultures is to establish a standard screening procedure for comparison of the rates of metabolism of different xenobiotics in plants (59-61). The metabolism rates could be ranked, and compared with other types of test results to establish a broad basis for the prediction of behavior of the xenobiotic in the ecosystem. Presently, there is no standard procedure for ranking xenobiotics on the basis of degradation in plant systems.

Although the rate of metabolism of a chemical is dependent upon the plant system in which it is placed, a standardized screen with certain plant species would provide a basis for initial comparison, which could be modified as more specific results were obtained. Proponents of a standardized method for comparison of xenobiotic metabolism in plant cell cultures have developed a flow diagram for such a screening procedure using soybean and wheat cell suspensions (Figure 6) (59). Using this procedure, the authors compared metabolism of di-(2 ethylhexyl)-phthalate, (DEHP), pentachlorophenol (PCP), 2,4-D, and mono-linuron in the cell cultures. Each of the compounds were chosen as models to represent a specific type of chemical. Mono-linuron and 2,4-D were chosen as important selective herbicides of differing modes of action; PCP was selected as a test chemical because of its ubiquitous distribution in the environment and because it represented an intermediate compound in terms of its hydrophilic or lipophilic nature. DEHP was chosen as a non-chlorinated lipo-

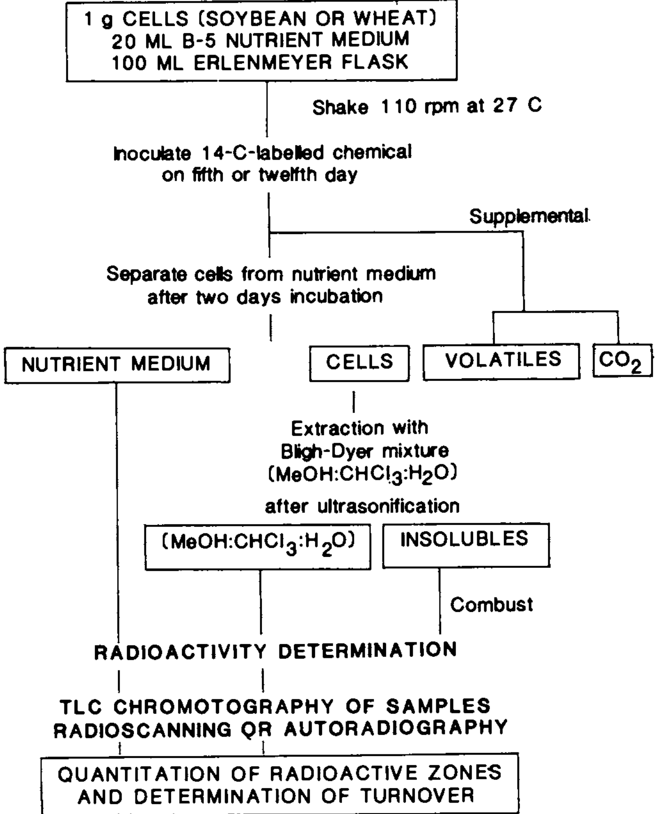

Figure 6. Flow diagram for determining the metabolic fate of chemicals in plant cell suspension cultures. (Reproduced with permission from reference 59. Copyright 1984, Pergamon.)

CHEMICAL	BIOACCUMULATION ALGAE	BIOACCUMULATION FISH	BIOACCUMULATION ACTIVATED SLUDGE	RETENTION in RATS %	BIODEGRADATION by ACTIVATED SLUDGE % CO_2	PHOTO-MINERALIZATION % CO_2	METABOLISM in PLANT CELL CULTURES %
Di-(2-ethylhexyl)-phthalate							
Pentachorophenol							
2,4-Dichloro-phenoxyacetic acid		~10					
Monolinuron		~10					

Figure 7. Comparison of metabolism of chemicals in plant cell cultures with other procedures for determining its behavior in the environment. (Reproduced with permission from reference 59. Copyright 1984, Pergamon.)

philic chemical. On the basis of the data obtained from these experiments, the authors obtained a ranking for the xenobiotics based on their metabolism in the cell cultures. The ranking was mono-linuron (17.6%) < DEHP (23.0%) < 2,4-D (57.2%) < PCP (83.1%). These results were compared with those of other test systems to provide another perspective on the potential behavior of the chemical in the environment (Figure 7). Although standardization of techniques for the use of plant cell cultures in pesticide metabolism studies is in its infancy, its greatest potential may be as a rapid assay to determine relative rates of metabolism of pesticides in a standard array of crops and important weed pests. In view of the series of standarized toxicological tests prescribed for potential pesticidal chemicals, it is not inconceivable that such a system could be implemented.

Summary

In general, studies of pesticide metabolism in cell cultures have shown that metabolism is qualitatively similar to that of the whole plant, but quantitative differences do exist. Whole plants or plant parts need to be used to confirm the quantitative aspects of pesticide metabolism observed in plant cell cultures. However, cell cultures can be used to estimate the phytotoxicity and metabolic fate of chemicals that exhibit poor uptake and mobility in whole plants. Thus, they provide an important adjunct to whole plant studies. In addition, higher yields of minor or transitory metabolites can usually be achieved in cell cultures, allowing the determination of a sequence of metabolic steps in a reaction.

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Chapter 3

In Vitro Plant Cultures for Herbicide Prescreening

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Cell cultures are ideal axenic physiological systems to study herbicide action without problems of cuticular transfer or complications of translocation. Still, not all metabolic systems function in all cells at all times in the cell cycle. Inhibitors of photosynthesis are often inactive in non green cells, and root-active herbicides may be degraded in green cells. Nutritional components in the medium may interfere with herbicide action. The criteria needed to develop universal pre-screens which show activity for all herbicides are discussed and evaluated. It is far easier to develop "dedicated" pre-screens which are used to measure single types of activity or single chemical types of herbicide. All results with *in vitro* systems should be validated with whole plants.

The major part of this chapter ostensibly discusses the use of *in vitro* systems for herbicide screening. The importance of this discussion exceeds that of screening, as the basic question addressed is the applicability of *in vitro* systems for use in all herbicide research. If *in vitro* systems do not parallel the plant response, there are great limitations to their use in metabolic studies in selecting for herbicide resistant crops, herbicide physiology studies, as well as screening. This chapter discusses advantages and limitations, successes and failures of using *in vitro* cultivated callus, suspension culture and *in vitro* mini-plant systems for universal and dedicated pre-screens. Various aspects of such systems have recently been reviewed (*1-3*). At least one commercial laboratory offers an *in vitro* pre-screen system (*4*).

The Need. Innovative pesticide producers typically screen five to twenty thousand chemicals per year per company for herbicidal activity. Such "primary" screens usually contain 5 to 10 plant species treated both pre and post emergence at one to three rates. The last published cost analysis for such a screen was 13 years ago and was

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$200 per compound (*5*). Less than 5% of the compounds tested are promoted to the next level of screening. The greenhouse space and the 4-8 weeks needed to obtain answers clearly call for quicker, cheaper pre-screens which can eliminate inactive compounds. Pre-screens allow for synthesis of milligrams instead of the grams of each analog to be screened. An *in vitro* pre-screen costing a tenth the cost of greenhouse screen need eliminate only 10% of the inactive compounds to break-even financially. Usually 40-60% of the inactive compounds are eliminated by *in vitro* pre-screening. In one series of thiocarbamates reported, only 35% of the whole plant inactive compounds could be eliminated when a 67% inhibition criterion was used(*3*).

The most important criterion for a universal *in vitro* herbicide pre-screen is that an active herbicide not be missed. This is not to say that whole plant screens have not missed active herbicides. Compounds have been tested and rejected as inactive that competitors later found and developed into major herbicides. Some industrial whole-plant primary screens were of too short duration to detect and assess the potential of herbicides such as glyphosate. As industrial chemists are intent in discovering "new-chemistry" i.e. new leads into novel herbicide chemistry - the imperative operational criterion should be restated as: an *in vitro* pre-screen must "not miss new chemical leads". The main thesis of this review is to show that *in vitro* systems are actually better able to meet this modified criterion than whole plant systems.

Advantages of in vitro systems. Considering that a pre-screen is used to elucidate phytotoxic or phytostatic compounds which are *potential* herbicides, they have the following advantages:

1. Cell cultures are axenic; they lack microorganisms of soil and leaf surface which might biodegrade a phytotoxic compound. This *can* be a double-edged sword; they also lack microorganisms that may activate a herbicide, although such are not yet known.
2. They lack a cuticle. The cuticular penetration barrier prevents ascertaining whether many new chemical groups are toxic. This barrier can later be overcome by synthesis of analogs of active structures or by better formulation. The knowledge that a chemical is toxic if it reaches the plant cell is probably the most important lead available from cell cultures that whole plant screens lack. The absence of cuticle and short diffusion distances in cell cultures, provide a possibility for performing rapid kinetic, physiological and biochemical studies (*6,7*).
3. Only small amounts of chemical are needed. Modern synthetic chemistry allows derivative synthesis of analogs and verification of their structure in small batches. If milligram quantities can show potential toxicity why synthesize grams?
4. Space requirements and labor are reduced. One technician, with racks of tubes or cluster dishes can screen a few hundred compounds per week. Cell cultures are pipetted, simple metabolic tests can be used for assessment and many steps can be automated.
5. Time. The longest *in vitro* pre-screen can give the chemist an answer within a week, if a compound has potential. This rapid feedback eliminates the frustrations of waiting months till the latest synthesis is given preliminary evaluation.

6. *In vitro* screens with metabolic measurements can provide more accurate numerical data for QSAR (quantitative structure activity relation) analyses.

Disadvantages. There are cases where there may be no advantage to *in vitro* systems:

1. *In vitro* pre-screens do not show up many compounds with plant growth regulator (PGR) potential. Many companies now consider PGRs as uneconomical and this may actually be an advantage.
2. Compounds can be missed unless extreme care is taken in designing a universal screen.
3. QSAR data are derived only for the cell level. Separate QSAR data must be obtained for penetration at the whole plant level. This too can be an advantage.

Balance. The major use of *in vitro* systems is clearly to "weed out" totally inactive compounds. They are not very useful for "look-alike" chemistry around existing active herbicides except where QSAR data at the cellular level are needed. Then one needs not use an "universal" pre-screen but a "dedicated" one for the typical particular chemistry involved. The main use for the "universal" pre-screen is screening from "random" syntheses and from directed syntheses for new chemistry. Dedicated screens should be used for "biorational" syntheses; syntheses designed to inhibit a specific metabolic pathway in the plant. Still, a compound synthesized to inhibit one pathway may instead inhibit another... and thus, should also be tested in a universal pre-screen.

Developing Universal and "Dedicated" Pre-Screens

No single system can be universal in showing up the herbicidal potential of all compounds. No *in vitro* system using cells or mini-plants represents all tissues and all species. Herbicides can be tissue specific and species selective and different herbicides act at different rates. This requires that any universal pre-screen has to be multi-specific, multi-tissue and multi-time. Many of the factors for consideration in developing such screens may superficially seem mundane or clearly apparent. Based on failures, this is only so in retrospect.

Evaluating Plant-Culture Parallelism. As there are many differences between spraying a plant post-emergence or the soil pre-emergence and treating *in vitro*, it is rather hard to directly correlate the two systems. The common procedure for evaluating the parallelism is to treat cultures and plants with a wide variety of herbicides, other pesticides and some non-toxic compounds. Two methodologies can be used to assess the parallelism: (1) rough criteria to show toxicity; (2) statistical ranking procedures.

The rough criteria indicate that if a compound is above a certain level of toxicity, then a compound should be screened. Using a single-culture procedure with non-photosynthetic cells, 3 of 19 known herbicides did not meet the promotion criteria (*3*). When a two-culture system was used, all herbicides tested would be promoted (*4*). and Table VII in (*1*).

Ranking procedures allow for more careful evaluation of parallelism. A series of compounds are put through plant and *in vitro* tests at fixed concentrations and they are ranked in both as to their relative activities (Figure 1). In any given system there can be false positives; those compounds that are more active *in vitro* than in plants (Figure 1a), usually because of the lack of the cuticle. False positives provide previously unknown information on potential phytotoxicity. Many non-systemic fungicides give positive responses in cell cultures (*1,4*). False negative compounds (Figure 1a) show no activity in culture but are active on plants. When a single culture system is used, there can be a good overall correlation despite false negatives (Figure 1c). Still, a false negative is a "missed" herbicide, and as such is unallowable. For this reason, multi-system pre-screens have been developed (*1,4*) to preclude missing compounds.

Much better correlation coefficients are usually obtained with single system dedicated pre-screens. These entail QSAR relationships using related compounds on a single plant *in situ* vs. *in vitro* pre-screen (Figure 1d), or use crop varieties having differential tolerance to a single herbicide (Figure 1b).

Choosing the Biological Systems. From the preceding discussion it is clear that more than one biological system must be chosen. Considerations for the ease of pipetting and handling have brought algae to mind. One such screen used Erlenmeyer flasks and required long incubations (*11*). Earlier literature (*12*). clearly shows that algae are efficient at showing activity of photosynthesis inhibiting herbicides such as prometryn and fluometuron (Table I). Algae are clearly inadequate at showing activity of phenoxy-type herbicides such as 2,4-D. More tonnage of 2,4-D type herbicide is still used than other herbicides, so algae cannot be used as the sole test organism. Cyanobacteria (blue-green algae) are even less representative of plants. They lack a higher plant type nucleus, and thus dinitroaniline type herbicides which prevent the tubulin formation necessary for nuclear division are inactive (Table I).

Table I. Effect of Selected Herbicides on Algal Growth

species	Prometryn (triazine)	Fluometuron (phenylurea)	Alachlor (chloracetamide)	Profluralin (dinitroaniline)	2,4D (phenoxy)
	(% inhibition of growth rate)				
Chlorella (green)	93	82	59	41	4
Anabaena (blue-green)	100	100	1	0	4

Source: Representative data condensed from (*12*).

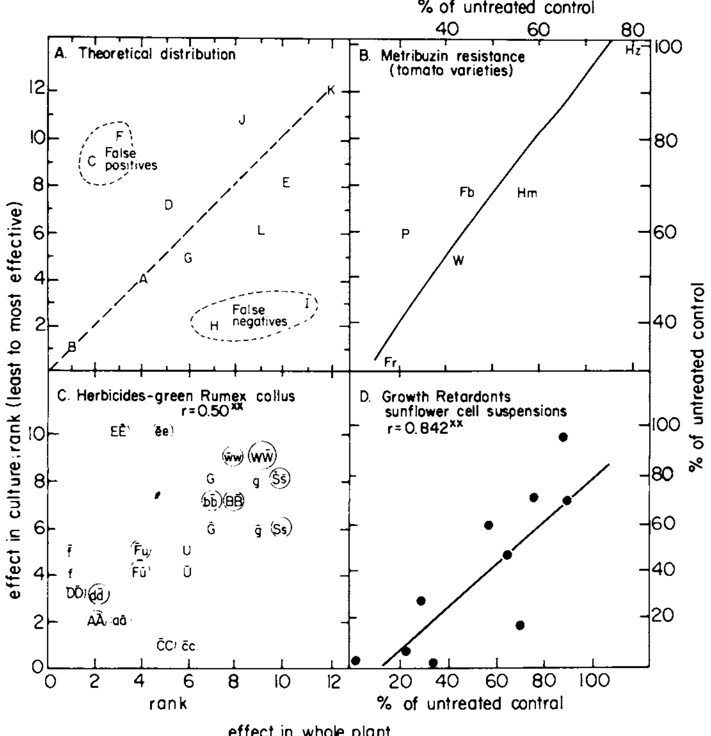

Figure 1. The effects of herbicides and inhibitors *in vitro* and in whole plants. A. The use of ranking procedures to correlate activity in whole plant and plant cell culture systems. Experimental compounds are ranked from least (1) to most active. B. Comparison of tomato varietal tolerances to metribuzin in callus cultures and seedlings. Metribuzin (0.25mM) was added to the medium of light-green calli. Seedlings were sprayed with 0.5 kg/ha metribuzin. The varieties are Fb, Fireball; Fr, Floridade; Hz, Heinz 1350; Hm, Homestead; P, Patriot; W, Walter. Drawn from data in Harrison et al. (*8*). C. The effects of various herbicides on growth of calli and seedlings of *Rumex obtusifolius.* Calli and seedlings were treated with two concentrations of each compound and the inhibitions of fresh weight were ranked from lowest (1) to highest (10). (A) daminozide; (B) 2,4-D; (C) dikegulac; (D) ethephon; (E) trifluralin; (F) napropamide; (G) simazine; (S) diuron; (U) chlorpropham; and (W) mecroprop. When more than one cross correlation has the same rank coordinates, the letters are circled. The figure is redrawn from data in (*9*). D. Effects of growth retardants in cell division of suspension cultures and on growth of sunflower plants. The PGRs include chlormequat and mepiquat chlorides and analogs, triazole types, AMO-1618 , daminozide, tetcyclacis and ancymidol. Redrawn from (*10*).

Algae clearly have their place in herbicide research. Their use in elucidating mode of action, especially at the molecular biological level in conjunction with genetics is well described in (*13*).

Species. Just as more than one species are used in a greenhouse screen, more than one species should be used in universal pre-screens. Not *all* selectivities that appear in whole plants will appear *in vitro*, as not all are based on metabolic parameters, so fewer need be used in a pre-screen. Even two seem sufficient from accumulated data. From experience, it would seem wise that at least one monocot and one dicot be included. One species is obviously enough if a screen is dedicated to finding a herbicide for a single problem species.

Tissue Type. Most cell cultures are white and thus are not inhibited by herbicides whose single mode of action is via photosynthesis (*9*). Thus, one tissue type must be green in a universal pre-screen. A green *in vitro* system is not sufficient as some herbicides, e.g. napropamide, do not inhibit green cells (*14*).

Measurement Time. The different modes of action of herbicides preclude developing universal pre-screens where only one time of measurement is used. Some herbicides kill rapidly, and measurements of toxicity can be made at almost any time (Figure 2, Line I). Some herbicides prevent synthesis of vital compounds. The lack of growth, starvation etc. can take a considerable time to be manifested (Figure 2, lines II, III). In some cases, where the herbicide prevents synthesis of compounds that turn over slowly (e.g. carotenoid biosynthesis inhibitors), the effects show up only in new tissue. One must thus wait until at least one doubling has occurred to see an effect. The effects of some herbicides that are phytostatic (i.e. stop growth) may wear off, and growth in culture may resume (Figure 2, line III). These may be effective selective herbicides as the unaffected crop continues growth, developing a canopy that starves the weed of light. One could miss such compounds by waiting too long to measure effects *in vitro.*

Media for in vitro systems. Many cell culture systems require complex media, which should be avoided as active compounds could be missed. Amino acid mixtures are often added to obtain rapid growth of cells. At least four groups of herbicides rather specifically inhibit different amino acid biosyntheses (glyphosates, sulfonyl-ureas, imidazolinones, glufosinate). They lack cellular activity when casein hydrolysate is used.

The differences in tomato varietal tolerance to metribuzin was thought not to carry over to cell cultures (*15*). These negative data were later shown to be due to high levels of sugars in the media, which antidoted the phytotoxicity (*8*). With lower levels of sugar, the correlation between varieties *in situ/in vitro* was very strong (Figure 1b).

Cellular Growth Phase. Many herbicides act at specific times in the cell cycle. The effect of dinitroaniline herbicides which prevent tubulin polymerization into microtubules can only be seen if there is

cell division. Herbicides which affect DNA synthesis will not show any effect in cells in stationary phase. Compounds affecting secondary wall biosynthesis will have little effect on rapidly dividing tissue. From these examples it is clear that a universal pre-screen cannot be developed using tissue in one growth phase alone. Indeed, systems utilizing isolated leaf cells (*7*). or protoplasts incapable of division, will miss many herbicides. The advantage of *in vitro* miniplant systems such as duckweeds is that the plants contain both dividing and stationary cells.

Evaluating Herbicidal Action. It is hard to simultaneously ascertain phytostasis and phytotoxicity. Besides the various quantitative measurements discussed below, visual observations are exceedingly important. Browning or bleaching of cultures can quickly tell much about the vitality of the cells. The appearance of new tissue after the herbicide is added can contribute much to the understanding of a compound's potential. Bulk physiological assays of whole samples can be inaccurate as they cannot differentiate between the effects of compounds on the small amount of new, and the lack of effect in the large amount of old tissue.

Growth (fresh and dry weight, cell number and packed cell volume) analyses can provide information about both phytotoxic and phytostatic herbicides. There is usually a good correlation between the effect of inhibitors on all of these measurements (*16*). The exceptions are telling: ethephon and chlormequat inhibit cell division but allow expansion and metabolite accumulation. Cell number is decreased but other parameters increase. A very simple assay for growth using sedimentation in matched Erlenmeyer flasks has been reported (*17*). There is no need to risk contamination while removing aliquots nor are cumbersome side arm flasks needed for turbidometric assays.

Biophysical measurements such as conductivity and preloaded dye release are useful to measure efflux from cells (*6*), as is gas exchange to measure respiration or photosynthesis. Release of gases such as malondialdehyde or ethane is used in "dedicated" assays of herbicides releasing active oxygen species causing lipoxydation of membranes.

Metabolic methods based on precursor incorporation and "vital" staining are used to measure the extent of inhibition by herbicides. No method is totally accurate unless the cells are totally and irreversibly dead. They can be a reflection of the situation during the throes of death. If a herbicide suppresses the biosynthesis of a given amino acid, and the assay for phytotoxicity happens to be incorporation of that amino acid into protein, there will be a strong stimulation of incorporation while the cells are dying due to diminished internal pools.

Positive "vital" staining data are usually equated with "life", implying a cellular capacity to grow and divide. Mammalian red blood cells and plant cells heavily irradiated with X-rays for use as nurse cultures are vital by such assays, yet they lack the ability to divide. Many vital stains, such as fluorescein diacetate and neutral red, measure the intactness of the cell membrane; tetrazoliums measure mitochondrial reductive capacity. Respiration continues and membranes remain intact well after "nuclear death". Some cells can be rescued after membrane damage and leakage. At best, lack of vital staining is indicative that cells are dying or are dead. Positive

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vital staining indicates, at best, that cells are not yet dead. This must be remembered at all times to prevent artifactual designation of toxicity.

Rather good correlations between procedures were obtained in comparing large number of compounds (*9*). Still, in developing a universal pre-screen, no toxic compounds may be missed. Data are summarized in Table II that accentuate the differences in various techniques in showing phytotoxic effects. Thus, more than one technique for measuring toxicity is needed in developing the universal pre-screen.

Table II. Rare examples of non-correlation among phytotoxicity measurement techniques in *Solanum nigrum* cultures

Inhibitor	fluorescein diacetate "vital" staining	tetrazolium reduction	leucine incorporation
	(% inhibition)		
0.1mM cycloheximide	88	19	81
1mM diuron	96	91	25
1mM diallate	84	62	7
1mM 2,4-D	92	40	84

Data of Zilkah and Gressel (*16*).

Additionally fluorescein diacetate staining showed little inhibition when growth was suppressed by simazine, asulam and chlormequat in *Cirsium* cell suspension cultures (*16*).

Other Potential Problems in Universal Pre-screens. Some soil active herbicides are highly volatile and there are problems in keeping them in the media for use in herbicide studies. This problem was overcome by using a non-volatile (non-commercial) thiocarbamate in cell culture studies to screen for herbicide resistant strains (*18*). This solution to the problem of volatility does not apply to universal pre-screens.

Many herbicides are highly apolar and thus the problem of water solubility may seem to be a problem. This is often overcome in cell suspension systems by spreading the herbicide dissolved in a volatile organic solvent on the inside of the culture tubes before adding the cells. The cells are added after the solvent evaporates and the herbicide partitions through the medium to the cells under the conditions of shake culture. Many cells can withstand 1% methanol and methanol soluble herbicides can be added in that manner. Conversely, ethanol is often phytotoxic.

Some herbicides such as paraquat have a tendency to be adsorbed to glassware (*19*). A finite amount is lost in saturating the glass

surface. This problem cannot be overcome by using plastic ware. Some highly apolar herbicides such as dichlobenil are readily absorbed or dissolved in plastics and can disappear from the media (*20*).

These problems of sufficient available herbicide can be partly solved by using supra-optimal herbicide concentrations. Usually 0.1 to 1mM solutions are used. These high concentrations can lead to false positives.

Conclusions on Developing a Universal Pre-screen. Universal pre-screens have been and can be developed which will not miss the vast majority of herbicidally active compounds. Fosamine-ammonium was missed in an *in vitro* pre-screen (*21*), but it is also missed in most greenhouse screens. This slow acting compound is sprayed in early autumn and its effect is only evident in the following spring. The pre-screen must be developed with many partial redundancies in technology so as not to miss potential herbicides. Thus there should be at least two of the following in a screen:

1. Phylogenetically unrelated species
2. Times of measurement
3. Types of measurement
4. Tissue types (green vs. non-photosynthetic)
5. Phases of cell cycle

This does not require numbers as large as might seem to avoid missing herbicides. One could use a green monocot miniplant (such as a duckweed), visually measure growth at various times and then photosynthesis by carbon dioxide fixation as one system and have already met several requirements from the above list. If log phase, non green cell culture of a dicot is used, and a vital or metabolic parameter is used, then all other requirements are satisfied.

Dedicated Pre-Screens.

When a single *in vitro* system is used as a universal pre-screen, the correlation between the real world of the plant and the artificial world of the plant culture systems can be minimal (Figure 1c). High correlations can and should be achieved with pre-screens dedicated to a single function. This is apparent from the studies such as that depicted in Figure 1d where growth retardants representing 7 chemically diverse groups were compared for activity in sunflower suspension cultures and whole plants. Similar correlations between the respective cell cultures were high when compared to rice, corn and soybean shoots ($r=0.971$, 0.985 and 0.900, respectively) (*10*).

The data can be more "dicey" when cell cultures are expected to correlate with complex morphological phenomena. Camper and colleagues found a good correlation between anti-suckering activity of 16 dinitroaniline compounds and tobacco callus growth inhibition. There was no correlation between *in vitro* and *in situ* activities of 10 nitrophenyl hydrazines in the same assay. The data of four publications are summarized by Camper in Figure 8 of ref. (*1*). Studies designed to obtain an assay dedicated to screen the elusive plant growth regulators that "enhance growth" were neither successful nor did they fail (*1*). This is not the fault of the *in vitro* system; none of the purported yield enhancing compounds tested showed significant yield enhancing activity in the whole plant (cf. Figure 11

in ref. *1*). They also did not show activity when a special assay was designed to show increased "sink" activity by measuring sucrose uptake into stationary phase suspension culture cells (cf. Table III in ref. *1*.)

Dedicated Pre-screens for Protectants and Synergists. Some compounds such as activated charcoal have been used to physically protect crops from herbicide action. It is doubtful how successful *in vitro* assays would be for such compounds. The more intellectually and commercially interesting compounds are those that protect crops from herbicidal action by some biochemical/physiological mechanism (*22*). Preliminary studies have shown that corn cell cultures are protected from EPTC action by dichlormid. The mode of protection is probably the same in culture as in the whole plant (*23*).

Dedicated pre-screens for protectants would best be developed with *in vitro* cultures of the crop to be protected. This can be problematic with herbicides which affect photosynthesis when green photosynthetic cultures are not available. A pre-screen for protectants against diquat was set up using a duckweed mini-plant system (Figure 3). Such a system has the advantage that potential protectants can be seen by their effects on growth and color. They can also be quantified by measuring $^{14}CO_2$ fixation or by chlorophyll extraction (*24*).

Pesticide synergists or activators are better known among the insecticides where four compounds are registered for commercial use in that capacity. Only one compound, tridiphane, has been reported to have herbicide synergist properties at the whole plant level (*25*). We are presently using cell culture systems to assay potential inhibitors of specific metabolic detoxification pathways as herbicide synergists, with considerable success at the laboratory level (*26*).

Concluding Remarks

In vitro systems have many advantages over whole plants in screens. When one realizes the limitations and keeps them in mind, *in vitro* systems, can be powerful tools to:

1. Pre-screen for some plant hormone activities.
2. Pre-screen for activities of some types of yield enhancers (that exist mainly in theory at present) or of compounds that may alter assimilate partitioning.
3. Pre-screen for activity of phytotoxic and phytostatic compounds such as herbicides, allelochemicals, and toxins from microorganisms.
4. Pre-screen compounds for ability to elicit resistance to herbicides (protectants) or to synergize herbicide action.
5. Obtain structure activity relationships for series of compounds where penetration problems should be precluded.
6. Microassay for metabolites of phytotoxic compounds or to bioassay phytotoxicity during chemical fractionations.

The advantages and limitations of *in vitro* systems must be weighed in every case against using greenhouse screens or organelles for similar studies. Only rarely will plant cell cultures be found wanting in such a comparison; usually they will be better on an absolute scale or on a time-effective or cost-effective scale.

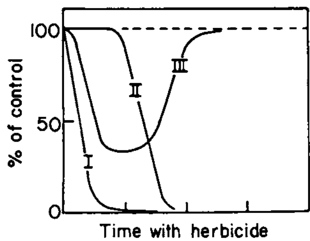

Figure 2. Examples of the kinetics of effects of different herbicides. I. A rapidly phytotoxic compound; II, an inhibitor which has a delayed action (e.g. it affects growth after one division or after depletion of a critical metabolite); III, a phytotoxic compound with a transitory effect,(the cells recover).

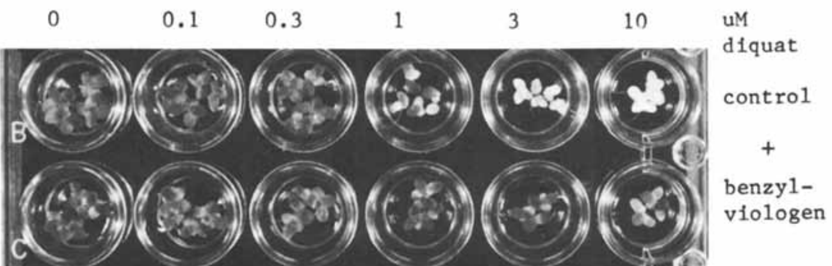

Figure 3. A miniplant system for pre-screening for herbicide protectants. Effects of benzyl viologen after 24h continuous co-treatment with diquat using *Spirodela oligorrhiza* as outlined in (*24*). Benzyl viologen also protects pea plants from paraquat (Shaaltiel, Gillham, Dodge and Gressel, unpublished results). Unpublished photograph of Lewinsohn and Gressel.

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The author is indebted to his students and colleagues S. Zilkah, P.F. Bocion, R.Levin, D.G. Davis, G. Ezra, E. Lewinsohn and Y. Shaaltiel who have vastly contributed to our research in developing the conceptual and data framework for designing pre-screens. The author is the Gilbert de Botton Professor of Plant Science.

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Chapter 4

Applications of In Vitro Selection Systems: Whole-Plant Selection Using the Haploid Phase of *Ceratopteris*

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Plant genes that confer tolerance to the herbicides paraquat and glyphosate have been selected for by using the haploid gametophyte phase of the fern *Ceratopteris*. The gametophyte generation of this plant, because it can be cultured in large numbers and is haploid, provides a convenient selection system for the induction and recovery of both dominant and recessive mutations. In contrast to plant cell culture or bacterial selection systems, this approach utilizes whole plants that are photosynthetic. Expression of the selected traits can be monitored in both haploid gametophytes and in diploid vascular sporophytes, which are produced by controlled self- or cross-fertilizations of selected gametophytes. This system provides a rapid and powerful means of identifying and characterizing a variety of single genes that may be beneficial for crop improvement through recombinant DNA techniques and for basic studies of plant molecular biology.

Crop improvement through genetic engineering is dependent upon the ability to identify genes that influence traits of potential agronomic interest. In addition, the availability of single gene mutations can be of major importance in basic studies of plant molecular biology. In recent years, the development of *in vitro* culture techniques has led to the successful application of microbial mutation selection protocols to higher plants (1,2). As a result, a number of plant genes of particular interest have been identified through this approach (3,4). Other approaches also offer promise for the identification of specific genes. Because the development of recombinant DNA technology is effectively circumventing natural barriers to gene exchange between species, virtually any organism may be a source of genes for use in genetic engineering or for basic research applications (5). Obvious possibilities include yeast, algal and bacterial systems which can be readily manipulated under laboratory conditions (6). Less traditional approaches also exist. For instance, recent studies with the homosporous fern *Ceratopteris*

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have shown that its free living haploid gametophyte generation can be effectively used to select for a wide variety of mutations that are of interest in the areas of plant molecular biology and crop improvement. Comparisons with more traditional approaches and specific advantages and applications of the Ceratopteris system are discussed below.

Higher Plant Systems

In vitro selection techniques have obvious advantages over the use of whole plants or seeds for the induction and recovery of genetic variants in higher plants. Mutation induction and selection at the single cell level avoids the complexities associated with multicellular systems and allows large numbers of individual cells to be screened simultaneously. The development and application of anther culture techniques in certain species has further improved the utility of this approach by allowing the selection and recovery of both recessive and dominant mutations from haploid cell cultures. As such, it has become possible to design higher plant selection systems that can effectively select for specific mutations that confer increased tolerance or resistance to pesticides, toxins, growth regulators and other chemical and physical stresses. In spite of these advantages, the induction and selection of mutants in cell or tissue cultures of higher plants also has certain technical limitations that hinder the successful general application of these techniques (1,7). These difficulties include: 1) the loss of regenerative capacity in older cell cultures that have been subjected to selection pressures and the consequent limit on the number of putative mutants that can be regenerated from such cultures; 2) problems in the recovery and maintenance of selected mutants because of genetic and chromosomal instabilities that frequently exist in cell cultures, even though such instabilities may be of direct value in generating genetic variants in the absence of any mutagenic treatment (8); 3) the inability to apply in vitro approaches to many higher plants and the consequent restriction of these techniques to relatively few genera; 4) the presence of cell aggregates in cell suspension cultures, which may result in the production of genetic chimeras that may subsequently interfere with the recovery and stability of certain mutants; and 5) selection at the level of undifferentiated and frequently non-photosynthetic cells, which can prevent the recovery of mutations that are expressed in whole plants or in photosynthetic tissue.

Continuing studies with higher plant systems are refining techniques and approaches so that many of these limitations are minimized. For instance, recent studies have shown that the use of protoplast cultures can successfully avoid many of the problems associated with cell culture systems (7,9). Because protoplasts obtained from green leaf tissue constitute genetically homogeneous cultures of single photosynthetic cells, the use of such cultures for in vitro selections is more likely to yield mutants that are expressed in differentiated tissues at the whole plant level. This attribute is essential where selection for resistance to photosynthetically active herbicides is of interest. In addition, because protoplasts are not maintained in culture for extended periods of time, genetic

instability and loss of regenerative capacity are not significant problems.

Alternative Approaches

Microbial systems provide more rapid and efficient selection systems than higher plant approaches and may therefore be a useful source for certain types of agronomically important genes. For instance, studies with the bacteria *Aerobacter aerogenes* (10) and *Salmonella typhimurium* (11) have resulted in the isolation and characterization of mutant strains that possess increased tolerance to the herbicide glyphosate (N - phosphonomethylglycine). The responsible gene in *S. typhimurium* has been identified and cloned. Long-range goals of this research include the incorporation and expression of this gene into higher plants in order to achieve genetically engineered herbicide resistance in selected crops (12). These approaches offer exciting opportunities in the area of plant genetic engineering. They also provide basic information about possible resistance mechanisms that may be selected for in higher plant systems. However, because selection is carried out in a bacterial system, the expression and potential agronomic value of the gene in a higher plant cannot be immediately assessed. This could be of considerable practical concern. For instance, certain mutations (see below), while conferring tolerance to a particular stress, may significantly reduce a plant's overall vigor and fitness. Such a situation could lead to an unacceptable "trade off" between tolerance and productivity, which would only be evident after a substantial commitment of time and resources was made in order to incorporate and achieve expression of the gene in a higher plant. In addition, microbial selection systems cannot be utilized for herbicides that are primarily active in photosynthetic tissues.

Alternative approaches to the use of higher plant cell or bacterial cultures in *in vitro* selections are possible through the use of organisms with different life cycle characteristics, such as the homosporous fern *Ceratopteris*. Studies with this plant have shown that because it possesses a free living haploid phase, features of both *in vitro* and whole plant selection can be combined to design an efficient means of selecting for a wide variety of mutations. In addition, particular features of both the gametophyte and sporophyte generations provide an exceptionally useful system for physiological, biochemical and genetic studies of selected mutations.

The life cycle of *Ceratopteris* consists of two independent autotrophic phases, a diploid sporophyte and a haploid gametophyte. The diploid vascular sporophyte phase can be readily cultured in the greenhouse and consists of plants with a short upright stem, associated roots and upright leaves ranging in length from 0.2 to 0.5 m. The margins of the leaves regularly produce sporophytic buds which can be detached, surface sterilized and cultured under laboratory or greenhouse conditions to produce additional plants. Mature sporophytes continuously produce large numbers of single celled haploid spores as direct products of meiotic divisions. Spores can be collected in essentially unlimited numbers and stored dry for several years. To establish gametophyte cultures, spores are surface sterilized and sown on agar-solidified or liquid medium containing appropriate macro- and micronutrients (13). Because the gametophyte generation is autotrophic and consists of intact plants, no carbon

source or growth regulators are needed in the medium. Germination of spores occurs within 5 days under continuous light at 25 C or within 3 days at 30 C. Sexual development in multispore cultures is moderated by a pheromone-like system involving the growth regulator, antheridiogen (14). All gametophytes in a culture have the capacity to develop into bisexual plants that can self fertilize when water is applied to their surface to liberate motile spermatozoids. These gametophytes, which are essentially two dimensional and heart-shaped, reach sexual maturity within 10-14 days from sowing, at which time they are ca. 1.0 mm^2 . Although gametophyte development proceeds normally in the absence of exogenously supplied growth regulators, the addition of growth regulators to the medium can dramatically alter development (15). For instance, the addition of abscisic acid to the medium alters sexual differentiation and significantly retards gametophyte growth (16). These responses have been used as the basis to select for abscisic acid-resistant mutants in Ceratopteris (17,18).

Because gametophytes are haploid and bisexual, self fertilization results in the production of completely homozygous sporophytes. In addition, gametophytes can be crossed readily to produce defined hybrid combinations in F1 sporophytes (18). Homozygous or hybrid sporophytes can be cultured and vegetatively cloned to provide an unlimited supply of genetically defined diploid vegetative material and haploid spores. The entire life cycle (spore to spore) can be completed in less than 120 days under laboratory and greenhouse conditions. These features of the Ceratopteris life cycle facilitate genetic studies and provide the means of designing an efficient and rapid selection system for the induction and recovery of specific mutations.

Haploid Fern Selection System

A selection system using Ceratopteris and capitalizing on many of the attributes of the life cycle discussed above has been developed for the purpose of recovering mutations affecting certain developmental processes or conferring tolerance to specific selection agents (17,18). Figure 1 illustrates the design of the screen. Single-celled haploid spores are mutagenized by exposure to X-irradiation and sown axenically on agar-solidified mineral nutrient medium containing the selection agent. Because of their small size, large numbers of spores (ca. 12,500) can be sown in a single 100 mm petri dish, allowing as many as 1.5×10^6 individuals to be screened in an area of ca. 1.0 m^2. Wild-type spores sown on medium containing an appropriate concentration of a selection agent (e.g. a herbicide or growth regulator) either fail to germinate or produce small abnormal gametophytes that frequently die within a short period of time. Any gametophytes containing mutations that confer resistance or tolerance to the selection agent develop normally and are evident as macroscopic green plants (ca. 1-2 mm diam.) usually within 21 days from sowing. These putative mutants can be scored visually and then transferred to individual culture dishes and self fertilized. The resulting sporophytes are homozygous for the selected mutation and can be cultured to produce unlimited quantities of genetically uniform M2 spores. To illustrate the utility of this selection system and its applicability to studies oriented toward the genetic improvement of

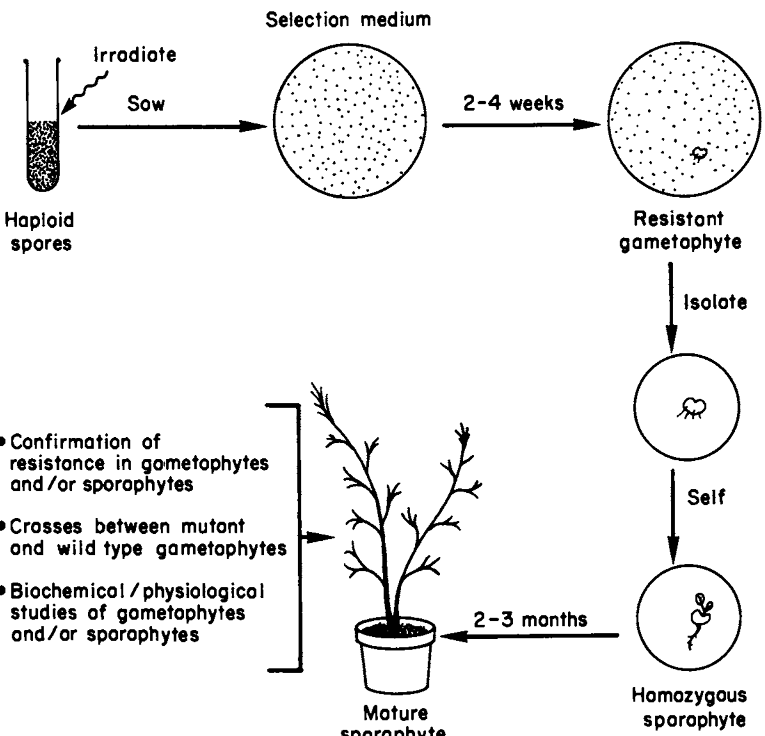

Figure 1. The Ceratopteris haploid selection system. Measurements of gametophyte areas were made with Bioquant image analysis system at 18 days following sowing. Data are represented as the percent of the size of each strain on the plain medium control.

crops, two examples of successful selections for herbicide tolerance are disscussed below.

Selection for Herbicide Tolerance in Ceratopteris

Paraquat Tolerance. Mutants of Ceratopteris tolerant to the nonselective herbicide paraquat (1,1'-dimethyl-4,4'-bipyridinium dichloride) were obtained from in vitro selections in the gametophyte generation using 0.5 μM paraquat as the selective agent (19). One hundred and thirty-eight putative mutants were recovered from the selection, at a frequency of ca. $2x10^{-5}$. To date, 19 of the selections have been tested for tolerance in the next (M2) generation and 17 were confirmed as stable mutants. Two of the selections were characterized extensively by comparative studies with the wild-type. Tolerance was assayed in both the gametophyte and sporophyte generations by monitoring chlorophyll retention in response to exposure to paraquat. In addition, tolerance was assayed in the gametophyte generation by assessing the effect of paraquat on growth. Tolerance to paraquat was evident in both gametophytes and sporophytes and the two mutants exhibited increased tolerance to paraquat of 10- to 50-fold over the wild-type. Although the two mutants were generally similar in their level of tolerance, it was possible to distinguish between them on the basis of slight differences in chlorophyll loss in both gametophytes and sporophytes and by comparison of growth inhibition in response to increasing concentrations of paraquat.

Subsequent studies (19,20) have shown that the two mutants are inherited as single gene nuclear mutations which are recessive in heterozygous diploid sporophytes. In addition, tests for complementation in the sporophyte generation have shown that the two mutants do not complement each other and are, therefore, functionally allelic. Extensive tests for recombinants in which ca. 1.25×10^6 gametophytes were monitored have not detected any recombination between the two mutations. Studies are continuing to further define the genetic basis of the mutants. For instance, spore populations derived from one of the mutant strains have been mutagenized and subjected to selection conditions of 2.0 μM, which is four times the concentration used in the initial selection protocol. A total of six highly tolerant putative mutants have been recovered from this selection and are being studied further (20).

The recovery and characterizations of the two paraquat tolerant mutants illustrate some of the advantages of the Ceratopteris selection system. Because a large number of spores can be screened, a large number of putative mutants can be recovered. Thus, it is possible to be highly selective in choosing which individuals to characterize further. For example, in the paraquat studies, individuals with morphological abnormalities or reduced vigor in the sporophyte generation were not investigated initially. The two individuals that have been fully characterized (see above) were chosen because of their slightly different responses to paraquat in initial growth tests and the possibility that they represented different mutations. Because gametophytes are haploid, it was possible to isolate recessive mutants. It is interesting to note that although considerable success was realized by using a basic diploid (n=x=39) stock of Ceratopteris, attempts to select paraquat tolerant mutants from an established polypoid (amphidiploid) strain with n=2x=78 were

unsuccessful, even though repeated attempts were made (20). The failure to recover mutants in these attempts may have been related to the inability to effectively select for recessive mutants in a gametophyte phase that was not truly haploid for the locus in question. Similar difficulties may arise in other non-haploid systems such as diploid cell or protoplast cultures and haploid cultures that are derived from amphidiploids, such as Nicotiana tabacum. It is also important to note that tolerant sporophytes were successfully recovered by selecting for tolerance in the gametophyte generation.

Paraquat's primary mode of herbicidal activity is associated with free radical production that is driven by electrons derived from photosystem I (21). Thus, if the tolerance mechanism is not specific to either sporophytes or gametophytes, one would expect both phases to respond in a similar manner, since both are photosynthetic. This situation contrasts with the apparent disparity between paraquat tolerance in selected calli and in subsequently regenerated sporophytes in selections from tobacco and tomato cultures (22,23). In both of these studies,tolerance was generally not evident in plants regenerated from selected calli, although calli derived subsequently from the regenerated plants were again tolerant to moderately high levels of paraquat. It is apparent that the selected calli, which were nonphotosynthetic and selected in the dark in the tomato study, did not possess mutations that were effective against paraquat's primary mode of herbicidal activity in photosynthetic tissue. A possible explanation of this behavior is that the callus or cell cultures were subjected to high concentrations of paraquat which, because the tissue was minimally or not at all photosynthetic, were only moderately toxic. Paraquat has been reported to exhibit moderate toxicity to cultures grown in the dark or that are nonphotosynthetic (24). As a consequence of this, when sporophytes were regenerated from this callus and tested for tolerance, their photosynthetic activity may have increased their susceptibility to such an extent that any tolerance selected for in the cell cultures would be effectively masked by the increased toxicity of paraquat in green tissue. As such, it would be expected that only mutations conferring high levels of tolerance would be detected by such a selection system and/or that tolerance expressed in both callus and regenerated sporophytes would involve altered uptake or transport of paraquat. In addition, because these selections were carried out in diploid systems, one would expect the recovery of only dominant or semidominant mutations, which is apparently the case in the tomato example (23).

Because the mechanism of action of paraquat in green tissue is reasonably well known and certain protective enzymes (e.g.superoxide dismutase (SOD), peroxidase, catalase) are possibly associated with tolerance to free radicals generated by the herbicide's activity (25-27), studies are being pursued to determine if any differences in the activity of these enzymes are evident between the wild-type and mutant strains of Ceratopteris. Initial studies have shown no differences in either constitutive levels of SOD, catalase or peroxidase, or in the uptake of labeled paraquat by whole gametophytes (28). This contrasts with studies of Harper and Harvey (29) in which elevated levels of SOD, catalase and peroxidase were detected in paraquat tolerant biotypes of Lolium perenne. However, recent studies by

Fuerst et al. (30) have attributed tolerance in biotypes of Conyza to exclusion of paraquat from its primary site of action in the chloroplast and not to any documented differences in enzyme activities. With the exception of the Ceratopteris examples, no well documented single gene mutations conferring paraquat tolerance in whole plants have been reported. The Ceratopteris mutants provide a well defined system with which to pursue further studies of their genetic basis and of the mechanism of of their tolerance to paraquat.

Glyphosate Tolerance. Studies have also been carried out with the broad spectrum herbicide glyphosate (N-phosphonomethylglycine). Selections for tolerance to glyphosate were conducted using a concentration of 0.1 mM glyphosate as the selection agent. Spores were mutagenized and sown on the selective medium and a total of ca. 450 putative glyphosate-tolerant mutants were recovered at a frequency of ca. $1x10^{-4}$. Preliminary studies of these selections have been carried out and are presented here.

A total of 131 putative mutants have been tested in the M2 gametophyte generation and of these 128 were tolerant to glyphosate. Maintenance of tolerance through a complete sexual cycle implies a genetic basis to the condition. Two of these mutants are further characterized here by comparison with the wild-type response of gametophytes to glyphosate-induced growth inhibition. Figures 2a and 2b illustrate dose-response data for the two mutants (G363 and G492) and the wild-type. Both mutants exhibit significant levels of tolerance to glyphosate when compared with the wild type. Figure 2a represents the data as a percent of the growth of each strain on plain medium. Both mutants and the wild-type are clearly distinguished. For instance, at 60% growth inhibition, strains G363 and G492 exhibit 5- and 15-fold increases in tolerance over the wild-type, respectively. Figure 2b illustrates dose-response data as absolute growth, expressed in mm^2. Again, differences are clearly evident between the wild type and the two mutant strains. It is important to note that although growth of the two mutant strains is superior to the wild-type at glyphosate concentrations greater than 0.03 mM, growth of the mutants at lower concentrations and on plain medium is less than the wild-type. Thus, it appears that the mutations conferring glyphosate tolerance significantly alter the growth potential of the selected strains on low concentrations of or in the absence of glyphosate.

Glyphosate tolerant mutants have also been obtained in the bacteria Salmonella typhimurium (12) and Aerobacter aerogenes (10). In cell cultures of carrot, stable glyphosate-tolerant lines have been obtained by long term culture on medium containing progressively higher concentrations of the herbicide (31). Glyphosate has been shown to be a potent inhibitor of the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSP; EC 2.4.1.19) in S. typhimurium (11) and A. aerogenes and the higher plant Corydalis sempervirens (32). Cell cultures of C. sempervirens and A. aerogenes that were exposed to increasing concentrations of glyphosate over time, became adapted to glyphosate at concentrations up to 10 mM (32). These adapted cells exhibited a 10 to 30-fold increase in EPSP activity. Similar results were reported in the stable carrot cell lines (31). In the carrot lines, it was noted that growth of the glyphosate-tolerant cells in medium lacking glyphosate was much slower

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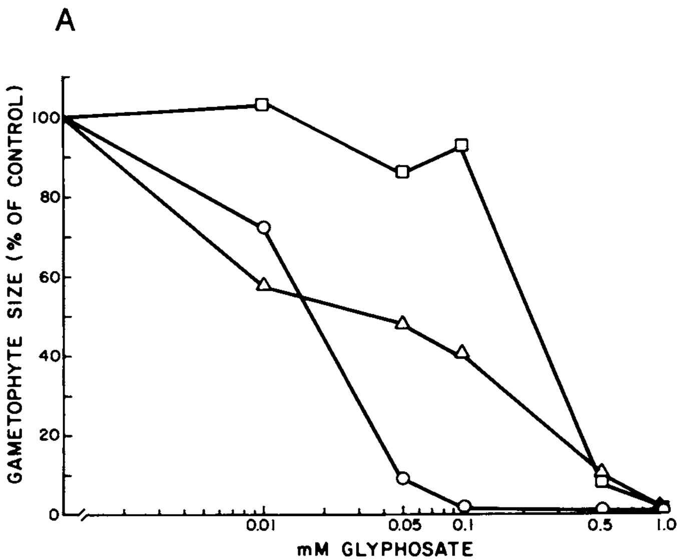

Figure 2 (A). Effects of glyphosate on growth in wild type (○) and mutant (G363 △, G492 □) gametophytes of Ceratopteris.

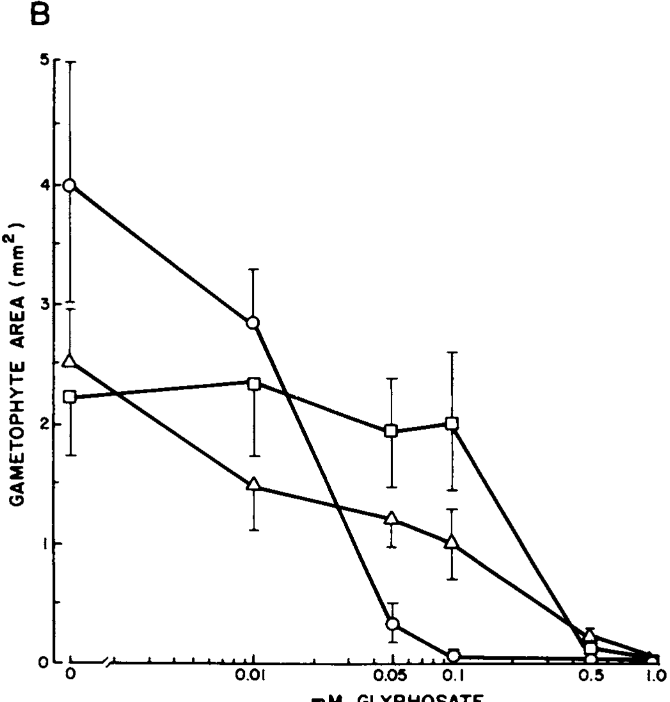

Figure 2 (B). Effects of glyphosate on growth in wild type (○) and mutant (G363 △, G492 □) gametophytes of Ceratopteris. Measurements of gametophyte areas were made with Bioquant image analysis system at 18 days following sowing. Data are shown as the actual areas of gametophytes. Each point represents the average of measurements from 20 individuals (±S.D. in B).

than nonadapted (selected) cells. This may be an effect similar to the one documented in the two Ceratopteris mutants.

Studies of glyphosate-tolerant strains have shown that EPSP activity is encoded by the aroA locus of S. typhimurium (12). Further studies have cloned a glyphosate-tolerant aroA mutant allele into E. coli and shown that the mutation acts as a dominant gene in merodiploids. Molecular studies have shown that the mutant allele contains a single base-pair change resulting in a structural alteration of EPSP, as well as a mutation residing in the promoter region of the operon (11). Tolerance in S. typhimurium appears to be associated with an increased affinity of EPSP for its substrates, phosphoenolpyruvate and shikimate-3-phosphate (11). Studies by Schulz et al. (10) have shown that a glyphosate-insensitive strain of A. aerogenes contains a glyphosate-insensitive EPSP.

Genetic characterizations of the Ceratopteris glyphosate-tolerant mutants have not been completed. From the initial dose response data, it appears that at least two mutations conferring different levels of glyphosate tolerance have been recovered. Further studies are needed to fully characterize the expression and secondary effects of the mutations in both the gametophyte and sporophyte generations and to investigate the biochemical basis of the trait.

Conclusions

The above examples of the use of the Ceratopteris haploid selection system serve to illustrate its utility for the isolation and characterization of single gene mutations for herbicide tolerance. Using this system, it is possible to initiate selections and determine if putative mutants have a stable genetic basis within a period of less than four months. During this time large numbers of putative mutants can be generated and initially characterized to identify potentially different mutations that confer various levels of tolerance. As such, the system is valuable for thoroughly investigating the genetic basis of selected mutations. In addition, because of the utility of the gametophyte generation for conducting growth studies under controlled conditions, it is possible to rapidly assess the effects of particular resistance traits on the growth and development of the organism. These studies can be subsequently extended to the sporophyte generation and conducted under greenhouse conditions. This capability can be especially important when selections are being carried out to identify mutations that might be of particular use in crop improvement. For instance, preliminary studies with the glyphosate-tolerant mutants described above indicate that the mutations conferring tolerance may also suppress gametophyte growth. If a similar effect is observed in the sporophyte generation, it would appear that tolerance associated with these particular mutations is not desirable from a genetic engineering perspective. The loss of growth potential in the glyphosate-tolerant carrot cell lines (31) may indicate a similar situation. Strains of S. typhimurium and E. coli. that contain a glyphosate-tolerant aroA allele exhibit a 15 percent lower growth rate on minimal medium than isogenic strains containing either a wild-type allele or both wild-type and mutant alleles (12). In contrast, tolerance in the A. aerogenes mutant did not effect growth potential, as expressed by the mean doubling time, in comparison with wild-type cultures (10). It is, of course,

impossible to assess the probable effects of such genes in a multicellular green plant until after their incorporation and expression.

Other attempts to employ homosporous ferns for the recovery of specific mutations have been reported. Carlson (33) used the ferns *Osmunda cinnamomea* and *Todea barbara* in a study involving indirect selection for auxotrophic gametophyte mutants. Howland and Boyd (34) selected for altered photomorphogenic responses to red light in gametophytes of *Pteridium aquilinum*. Both of these studies, however, were limited by the inability to culture the selections through an entire sexual cycle and were therefore unable to confirm a genetic basis for the putative mutants that were recovered. Few genetic studies have used ferns as experimental organisms. This may be associated with the widely held view that all homosporous ferns are derived from polyploids (i.e. paleopolyploids, see 35), contain numerous duplicated loci and are genetically complex (36). However, experience with selections using *Ceratopteris*, along with data from recent studies of isozyme variation within other ferns (37,38) show that extant diploid ferns generally behave as genetic diploids and not as polyploids. Therefore, ferns with appropriate life cycle features, such as those illustrated by *Ceratopteris*, can be of considerable use experimentally.

In addition to selections for herbicide tolerant genotypes with *Ceratopteris* , the selection system has also been utilized to select for a wide variety of other traits, including insensitivity to the plant hormone abscisic acid and tolerance to sodium chloride, L-azetidine-2-carboxylate, hydroxyproline, 5-flurodeoxyuridine and 2-aminoethyl-L-cysteine. The continued use of this system can provide many additional opportunities for the selection and recovery of a variety of mutations for research applications in plant molecular biology, herbicide chemistry and genetic engineering. As in any system, there are limitations - for one, *Ceratopteris* has not been established as and doesn't appear to have much promise as a major agricultural crop! However, the success to date with a wide variety of selection agents, along with the general utility of the system as illustrated with the above examples, indicate that the system provides certain experimental advantages for *in vitro* mutation selection and characterization. The continued development and application of molecular techniques can allow the practical utilization of selected mutations from this and other selection systems in both basic and applied studies.

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Chapter 5

Use of Plant Cells and Organ Culture in the Production of Biological Chemicals

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Plant cell and organ cultures can now be established for numerous species of higher plants, and may become in the future a commercial source for a variety of natural products (drugs, pesticides, flavors, and fragrances). A major block in realizing the industrial potential of plant cell cultures is our limited knowledge of secondary metabolism and its regulation. Recently developed systems may contribute to our understanding of the underlying regulatory mechanisms. Immobilized plant cells can produce larger amounts of natural products than batch suspension cultures (i.e., capsaicin from *Capsicum frutescens*), and can also be used in biotransformation reactions not easily accomplished by microorganisms (i.e., *Digitalis lanata* and *Catharanthus roseus*). Using amino acid analogs and other metabolic inhibitors as selective agents, cell variants overproducing secondary metabolites and/or their precursors can be obtained. In such systems simple nutritional switches may stimulate the production of a secondary metabolite. Differentiated fast growing root cultures can be established upon infection of dicotyledonous species with *Agrobacterium rhizogenes*. These transformed root cultures grow at higher rates than normal roots while showing stable production of secondary metabolites, in contrast to unorganized cell suspensions which fail to produce them (i.e., tropane alkaloids from the Solanaceae). These approaches are discussed from a basic and applied perspective.

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"O Tiger-Lily" said Alice, addressing herself to one that was waving gracefully about in the wind, "I wish you could talk!"
"We can talk", said the Tiger-Lily, "when there is anybody worth talking to."
(Lewis Carroll, *Through the Looking Glass*)

Higher plants have evolved an extraordinary variety of secondary metabolic pathways, the resulting products of which have been put to use by man providing pharmaceuticals for drug use, insecticides and various allelochemicals for pest control, and extracts for the flavor and fragrances industries. In spite of advances in synthetic organic chemistry, plants remain a major source of natural products, particularly in the specialty chemicals industry. Compounds, such as the insecticide derived from *Azadirachta indica* or the antitumor alkaloids vinblastine and vincristine found in periwinkle (*Catharanthus roseus*) (1), have complicated structures which preclude at the present time the development of an economical chemical synthesis (Figure 1). In the case of flavor (2) and fragrance (3) components, organic chemists would also be hard pressed to reproduce the nuances of plant cell chemistry. It is therefore not surprising that a significant amount of research has focused on ways to manipulate the production of plant natural products above the levels normally found in the plants. Economic benefits are an obvious incentive behind these efforts. The periwinkle alkaloids, which are the most effective chemical therapeutic agents for various forms of leukemia, have a retail price of over $6,000/gram (4). Most important, however, is the fact that only about 10% of an estimated 750,000 species of higher plants have been surveyed in any detail for biological activity (5). At the current rate of destruction of tropical rain forests, where a majority of species are found, it becomes urgent to study and develop novel ways to preserve and understand this chemical potential before it is lost forever. New experimental systems and their related biotechnologies may contribute significantly to this endeavour.

The pioneering work of Nickell and coworkers at Charles Pfizer Co. in the 1950's started the interest in the industrial applications of plant cell cultures. Cell suspensions of Paul's scarlet rose, grown as small aggregates, were raised in carboy fermentors at volumes of up to 134 liters in a defined culture medium (6). In spite of their obvious differences with microbial cells relating to size (20-100 μm), water content (60-90%), and generation time (20-40 hrs), plant cells were eventually grown using fermentation techniques that had long been routine for microbial cells. *Nicotiana tabacum* cell lines, for example, have been grown in 20,000 liter tanks at a specific growth rate of 1.09 per day (7). Early in these studies it became apparent that plant cell cultures might be used to produce secondary metabolites, and this resulted in the patent by Routien and Nickell (8). A considerable effort in this direction was made in laboratories in Japan and Germany (9), and to a lesser extent in the U.S. In 1983, the first plant cell culture process was commercialized in Japan.

Shikonin

Hyoscyamine

Vinblastine

Nicotine

Berberine

Digoxin

$CH_3CH_2CH_2CH{=}CHCH_2CH_2CH{=}CHCONHCH_2CH(CH_3)_2$

Pellitorine

$H-\text{(thienyl)}-\text{(thienyl)}-C{\equiv}C-CH{=}CH_2$

Dithiophene

Figure 1. Chemical structures of various natural products found in plants. Dig = Digitoxose.

Production of Shikonin Derivatives

Extracts from the root of Ko-shikon (*Lithospermum erythrorhizon*) have long been used in Japan as dyes and for their anti-bacterial activity in the treatment of wounds, and burns. The active principles, a series of anthraquinone derivatives known as shikonins, accumulate in the root to a level of 2% (dry wt.). The total supply of Ko-shikon is imported from China and Korea and it takes the plants 5-7 years to grow to commercial size. Pure natural shikonin sells for about $4500/kg. Based on an earlier report showing shikonin production in root derived callus cultures (10), Fujita et al. at Mitsui Petrochemical Ind. developed a method for the commercial production of these compounds (11). A cell line producing 12-15% dry cell weight of shikonin was selected, a process facilitated by the fact that high producing cells are bright red. Following the approach developed by Zenk et al. (12), two well known culture media formulations were adapted to allow for rapid cell growth followed by accumulation of the compound in a two-stage process (11, 13). KNO_3 as a sole nitrogen source, phosphate (1 mM), and Cu^{++} (0.5-2.0 μM) were required for maximal shikonin production in a modified White's medium (M9). This two-stage protocol was scaled-up in a tank air-lift system (first st.; 200 l: second st.; 750 l), to achieve a final accumulation of up to 23% dry weight shikonin, with an average of 14% after each 3 week growth/production cycle (11, 13). The profile of compounds formed in cell suspensions was similar to that of the Ko-shikon root. This fermentation system is now the major commercial source for shikonin (13).

The production of shikonins is an example of both the promise and limitations of current plant cell culture procedures in the area of natural products. While productivity at least 10-fold higher than in plants was obtained, this was the result of a systematic but empirical search for the optimal conditions for shikonin accumulation. In many cases it has not been possible to find the culture conditions appropriate for the expression of a secondary metabolic pathway (14, 15). About a dozen examples are known of cell cultures producing a secondary metabolite at levels equal to or higher than in the whole plant (Table I), but with the exception of shikonin none of these systems has commercial use, or is competitive with existing extraction technology.

It is generally agreed that in order for a cell culture system to be commercially viable, the market value of the compound should be at least $500/lb (13). Given the relatively slow growth rates of plant cells, the particular compound(s) would have to accumulate to at least 2-5% of the cell dry weight. Important compounds, such as the morphine alkaloids, cardiac glycosides, tropane alkaloids, anti-tumor alkaloids, and essential oils, cannot yet be produced in cell suspensions at levels even approximating those of the plant (13, 15, 16). The main reason for this situation is our meager understanding of the regulation of secondary metabolism. Recent advances, however, make it likely that some of the questions concerning secondary metabolism in higher plants may soon be answered. Some of these approaches will be briefly discussed below, including the use of immobilized and

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Table I. Natural products accumulated in high levels by plant cell suspensions

Compounds	Plant Species	Yield	
		g/l	% dry mass
Rosmarinic acid	*Coleus blumei*	3.6	15
Verbascoside	*Syringa vulgaris*	1.4	16
Cinnamoyl Putrescines	*Nicotiana tabacum*	1.0	10
Anthraquinones	*Morinda citrifolia*	2.0-2.5	15-18
	Galium mollugo		
Shikonines	*Lithospermum erythrorhizon*	1.4	12
Benzylisoquinolines	*Berberis stolonifera*	2.7	15
	Coptis japonica		
Benzophenanthridines	*Eschscholtzia californica*	0.15	1.7
Nicotine	*Nicotiana tabacum*	0.1	2.5
Ajmalicine	*Catharanthus roseus*	0.3	1.0
Serpentine	*Catharanthus roseus*	0.2	0.8
Diosgenin	*Dioscorea deltoidea*	0.05	0.5
Ubiquinone-10	*Nicotiana tabacum*	0.05	0.5

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biotransformation systems (17), the use of cell cultures overproducing metabolic intermediates (18), and the development of plant organ culture (19). The reader is referred to recent reviews for a more complete overview of this field (9, 20, 21, 22).

Immobilized Plant Cells and Bio-transformation

Methods for the immobilization of viable plant cells (IPC) have received recent attention in view of their potential for the study of secondary metabolism. These techniques are derived from those used for microbial cells and generally involve embedding the cells in a polymeric matrix, such as gels of calcium alginate, agar and polyacrylamide) (17). While the effects of the immobilizing material on cell viability and metabolic activity vary widely, it is generally agreed that such systems provide certain advantages over conventional batch or continuous cell suspension culture. First, what is known about secondary metabolite formation in suspension cultures suggests that this process is generally not directly coupled with growth (see section on organ culture). It would therefore seem that bioreactor systems that do not promote growth could be more efficient for secondary metabolite production. In theory a greater percentage of nutrient flow would be diverted from biomass formation into a specific metabolic pathway. In addition, an immobilized system would simplify a continuous production system and allow reuse of the plant cells.

An improved technique for entrapment of plant cells has been recently developed with *Capsicum frutescens* cell suspensions. Cells were shown to passively invade the pores of a reticulated polyurethane matrix, inside which viability and metabolic activity were better preserved than in other immobilizing matrices (23). This sytem was used in a detailed study of the synthesis of capsaicin (an ester of vanillylamine), which is the pungent principle of chili pepper fruits. In the absence of specific precursors to capsaicin, the entrapped cells produced 2-3 orders of magnitude higher yields than cell suspensions over a 5-10 day culture period (4-5 mg capsaicin g^{-1} dry weight l^{-1} versus 30 μg g^{-1} l^{-1}). An inverse relationship between incorporation of ^{14}C-phenylalanine (Phe) into protein or capsaicin was shown, and this was reflected by an increased rate and amount of incorporation of labelled Phe into capsaicin in entrapped as compared to freely suspended cells (23). Capsaicin accumulation could also be increased by supplementing the medium with precursors, such as Phe and isocapric acid, or by decreasing the growth rate through omission of growth regulators from the culture medium. Although the reasons for the increased productivity of entrapped cells are not completely clear, the following factors have been cited (24, 25): (a) close cell contact is encouraged in entrapped cells, thereby limiting cell division rate and allowing the establishment of gradients in nutrients and other factors important for the expression of secondary metabolism; (b) manipulation of the medium is facilitated in entrapped cells cultured in a bioreactor; and (c) collection of the product can be achieved without biomass destruction if the product is released into the medium.

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Another use of IPCs involves one or two step biotransformations such as hydroxylations, glycosylations, acetylations and methylations. The most detailed work in this area has been on the hydroxylation of cardiac glycosides of *Digitalis lanata* and the formation of indole akaloids in *Catharanthus roseus*.

Digoxin and its methyl analog (Figure 1) are widely used in the treatment of heart diseases. In the plant, digoxin is derived from its precursor digitoxin from which it differs only by an additional hydroxyl function at C-12. It has been shown that *Digitialis* cell suspensions could directly transform exogenously added methyldigitoxin into methyldigoxin (26). Alginate entrapped cells were able to hydroxylate methyldigitoxin much more efficiently than suspended cell cultures. After an initial lag, transformation was found to occur at a constant rate for up to 170 days.

The synthesis of indole alkaloids from the common precursors tryptamine and secologanin by cell cultures of *C. roseus* has been studied in detail (17). Cells entrapped in agar, agarose, or carrageenan produce ajmalicine isomers at about the same rate as the freely suspended cells, while cells entrapped in alginate showed up to 160 fold increased synthesis. A major reason for this increased synthesis is believed to be the restricted growth of the entrapped cells, as has been discussed above for *Capsicum*. The yield of ajmalicine is above 12 times as much product formed from added precursors as by de novo synthesis. In this case too, alginate entrapped cells were much more productive than freely suspended cells (140% increase), and were also used to synthesize the related alkaloid serpentine in a batch procedure (17).

A major requirement for the application of IPCs in secondary metabolite production and biotransformations is that the plant cells release the products to the culture medium. In *Capsicum* cells, capsaicin released into the medium accounts for 99% of the total yield. Similarly, the cardiac glycosides and indole alkaloids transformed by *D. lanata* and *C. roseus* cell suspensions are released into the medium. An extreme example is provided by cell suspensions of *Thalictrum minus* producing berberine, an isoquinoline alkaloid used as an intestinal antiseptic (Figure 1). Most of the berberine produced by these cells was released continuously into the liquid medium, an excess of which crystallized as the nitrate salt (27).

Unfortunately the majority of secondary metabolites produced by plant cells are accumulated in the vacuole or elsewhere in the cell. It is therefore necessary to devise methods for selective permeabilization and controlled release. Dimethylsulfoxide (DMSO) treatment allowed alkaloid release from *Cinchona ledgeriana* cells (28); however, the high degree of permeabilization obtained after 1 hour treatment with 15% DMSO resulted in permanent cell damage. Lower concentrations (5-6% DMSO) preserved cell viability, but permitted only limited release of the alkaloids. It is expected that increased knowledge of systems for secondary metabolite transport and storage will eventually result in the design of specific permeabilizing agents. Plant cell imobilization remains an attractive method for the production of plant chemicals, and may eventually lead to practical applications (29).

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Use of Metabolic Inhibitors as Selective Agents

Research done in the last 10 years by Widholm et al. has involved the selection of cell cultures resistant to amino acid analogs and herbicides. Stable cell lines carrying such resistance markers may be useful in genetic manipulation involving somatic hybridization and gene transfer. The best studied cultures have been carrot and tobacco suspensions selected for resistance to 5-methyltryptophan (5MT), hydroxyproline (HP), ethionine (Et), and p-fluorophenylalanine (PFP) (18). In most cases, the resistant line shows overproduction of the corresponding amino acid rather than decreased uptake of the analog. The first succesful selection resulted in carrot and tobacco cells resistant to 5MT which contained 30- to 40-fold higher levels of free tryptophan (Trp) than the wild type (30). Anthranilate synthetase, the first enzyme in the aromatic amino acid pathway leading to Trp, was shown to be less sensitive than the wild type enzyme to feedback inhibition by Trp and 5MT. Similarly, a carrot strain resistant to HP was also found to be resistant to another proline analog, azetidine-2-carboxylic acid (A2C) and contained 15-30 times normal proline levels. In this case, however, the enzymatic alteration was not studied. Recently (18), several tobacco cell lines resistant to Et were selected. These lines were able to tolerate 27-fold higher levels of Et than the wild type, and the free amino acid profile showed a dramatic increase in the levels of aspartate derived amino acids (methionine, 110X; threonine, 18X; lysine, 5X; isoleucine, 5X). Glycine and serine levels were also elevated. Aspartate kinase, the first enzyme common to all aspartate-derived amino acids, was elevated 9-fold in the resistant cells.

The above studies show that it is possible to select for an alteration in amino acid biosynthesis and to gain insights into metabolic control points by studying the resulting enzyme alterations. Such cell lines could also be used in the production of secondary metabolites. Since amino acids are precursors for numerous alkaloids and other natural products, it should eventually be possible to find conditions under which the free amino acid pools of overproducing cells could be efficiently diverted into the production of a taxon-specific metabolite. Alternatively, the selection process itself may result in a strain overproducing a secondary compound.

Berlin and Widholm (31) selected carrot and tobacco cells resistant to PFP. In contrast with the carrot line, which showed the expected increases in free Phe, the PFP-resistant cells (TX4) had almost normal levels of Phe, but did show dramatic increases in phenolics apparently due to a 10- to 20-fold rise in phenylalanine ammonia lyase (PAL) activity as compared to control cells (TX1). It was suggested that this cell line was resistant to PFP due to the higher PAL activity, which could detoxify PFP by conversion to p-fluorocinnamic acid. If this method could be generally applied, it would allow for the positive selection of cell strains overproducing a specific class of secondary compounds.

Further characterization of the TX4 cells showed that the main phenolic compounds were the caffeoyl-, feruloyl- and coumaroyl amides of the diamine putrescine (1,4-diaminobutane)

(32). The hydroxycinnamic acids (HCAs) of di- and polyamines are found in male and female flower parts of mono- and dicotyledonous species, but are absent from vegetative organs. From these findings and the strong correlation found between male fertility and accumulation of specific patterns of HCAs in anthers, these compounds have been suggested as having an important role in flower differentiation (33). A cell suspension, such as the TX4 line which shows a simplified HCA pattern, may be a convenient system for the study of biochemical events associated with flowering, as well as of secondary metabolism. Although the growth kinetics of TX1 and TX4 cells were similar, the latter contained between 5-10% cinnamoyl putrescines (dry wt basis) throughout their growth period in batch culture. This derepressed synthesis of HCAs in TX4 cells was due to increased activities of PAL, trans-cinnamate-4-hydroxylase, and p-coumarate-CoA ligase, all of which are involved in the phenolic pathway. Furthermore, ornithine and arginine decarboxylase, the alternative enzymes for putrescine biosynthesis, were also significantly increased in TX4 cells. Related experiments with the analog DL-meta-fluorophenylalanine also have resulted in the establishment of cell lines accumulating 2-5 times more cinnamoyl putrescines than the wild type strain (34), with the expected increase in PAL activity.

Nutritional Switches and Secondary Metabolism

Experiments on the inorganic nutrition of tobacco cells have yielded further insights on the regulation of the pathway leading to cinnamoyl putrescine accumulation. It was observed that inorganic phosphate was completely taken up from the medium by freshly inoculated cells within the first 2 days after transfer (35). Intracellular ortho-phosphate concentrations of up to 0.06 M were accumulated, and subsequently diluted by cell growth and division. If cells at different stages of the growth cycle containing progressively decreasing levels of phosphate were transferred to phosphate-free medium, cinnamoyl putrescines accumulated. The final level of these compounds was inversely correlated with the intracellular phosphate level, as opposed to the direct linear correlation between phosphate concentration and further growth in the phosphate-free medium. Thus, a nutritional switch can mimic the phenotype in secondary metabolites obtained upon selection for resistance to a metabolic inhibitor. This finding may find an application in controlling the level of other secondary compounds. Tobacco cells, which normaly produce only trace amounts of the alkaloid nicotine, will increase it 5-fold upon transfer to medium containing 1/10 of the phosphate level normally employed (36). Immobilized *Capsicum* cells similarly accumulated capsaicin when deprived of phosphate (25).

The induction of cinnamoyl putrescine accumulation in phosphate-depleted tobacco cells is maximal when inorganic nitrogen is supplied (37). This is not surprising since nitrogen is a component of the HCA precursors Phe and putrescine. However, the fact that ammonium citrate is much more effective than KNO_3 in the induction of cinnamoyl putrescine synthesis in

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phosphate-depleted cells suggests that a mechanism related to nitrogen assimilation is also involved in this phenomenon. While plant cells usually grow in the presence of NO_3^- and NH_4^+ supplied alone or in combination, it is less well known that growth is also possible in organic nitrogen sources. In addition to KNO_3, the standard nitrogen source, the XD cell line of tobacco has been adapted to grow on urea, gamma-aminobutyric acid (GABA) and arginine as sole nitrogen sources with a similar doubling time (2 days) (38). Putrescine pools were dramatically affected by the nitrogen source in which the cells were grown. In particular, XD cells grown on KNO_3 had very low levels of cinnamoyl putrescine, but when transferred to GABA, arginine, or in the cell line adapted to urea (14U cells), the amounts of these conjugates increased to 3-5% dry wt. Considering these cells are maintained in a N limiting regime (3 mM), such a dramatic diversion into a secondary metabolite suggests that cultured plant cells have a metabolic plasticity deserving of further exploration.

The above described experimental system also lends itself to the selection of metabolic pathways not found in the wild type cells. The XD strain is not able to grow in the presence of putrescine as the nitrogen source; instead, the cells accumulate putrescine as the cinnamoyl conjugates. By maintaining XD cells in putrescine containing medium for extended periods of time, it was possible to select a variant line able to metabolize the diamine very efficiently (19). These putrescine utilizing (PUT) cells converted the diamine to GABA, as shown by labeling experiments. In contrast, XD cells only converted putrescine into the polyamines or HCAs. PUT cells or GABA-grown cells were killed by nM levels of gabaculine, a suicide inhibitor of GABA transaminase (an obligatory step for the utilization of nitrogen in GABA), while XD cells grown on KNO_3 or urea were sensitive to a 1000-fold higher level of the inhibitor. Furthermore, PUT cells were more sensitive to amino-oxy phenylpropionic acid (AOPP), a competitive inhibitor of PAL and thereby of cinnamoyl putrescine accumulation. This suggests the putrescine conjugates are the intermediaries in the conversion of putrescine to GABA. In fact, pulse-chase experiments with ^{14}C-putrescine provide evidence for the turnover of cinnamoyl putrescine in PUT cells, but not in the XD line. In this respect, PUT cells behave biochemically like flower cells, since this is the only organ in the tobacco plant in which putrescine appears to be metabolized to GABA. We may recall that HCAs accumulate only in flowers, and there is recent evidence that these compounds are turned over during early fruit development (39). In summary, simple switches and selection based on inorganic and organic nutrition have a dramatic effect on the expression of secondary pathways in cultured cells.

Culture of Plant Organs

Two major generalizations can be made regarding the production of natural products in callus and cell suspension cultures. First, in undifferentiated cells, growth is usually incompatible with the activation of a secondary pathway (9, 20, 22). Even in the case of compounds produced by cell cultures at levels higher than in

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the whole plant (Table I), in the majority of cases this occurs during the stationary phase, or when cells are transferred from a growth medium to a production medium. An apparent exception is the formation of cinnamoyl putrescines in tobacco cells resistant to p-fluorophenylalanine (32). Second, the production of a secondary metabolite resumes after an unorganized structure is induced to undergo organogenesis or embryogenesis. For example, root-differentiating calli of *Atropa belladona* are capable of producing alkaloids such as atropine, while non-differentiated calli do not (40). Similarly, tropane alkaloids are produced in root-forming calli from *Scopolia parviflora* and *Duboisia leichhardtii* (41, 42) but only trace amounts in disorganized tissue. The cardiac glycosides from *Digitalis* can be formed when calli are induced to differentiate into embryos (43). Since in the whole plant secondary metabolites accumulate in specific tissues and organs during particular developmental stages, the failure by undifferentiated cells to produce secondary compounds is not surprising. Therefore, the study of continuously growing organized cultures may give clues as to how to unlink metabolic from morphological differentiation. In addition, these organized systems may themselves have potential as commercial sources of natural products.

In 1934, White (44) reported the establishment of tomato root cultures capable of unlimited growth. These were in fact the first "plant tissue cultures". For over two decades, established root clones grown in defined medium provided valuable systems for the study of inorganic and organic plant cell nutrition. By the early 1960's (45), numerous reports were available describing the growth of roots from mono- and dicotyledonous species in completely defined media. White's original root clones were grown for at least 25 years in liquid medium requiring sucrose, thiamine, and pyridoxine as the sole organic components.

A separate development established the fact that roots can perform specific secondary metabolic activities in addition to absorbing water and nutrients from the soil. In his classic studies of reciprocal grafting between tobacco and tomato, Dawson (46) provided the first solid evidence that roots were the major, if not the sole site of synthesis of the alkaloid nicotine. His findings were almost immediately confirmed using isolated tobacco root cultures (47). It was later shown that the tropane alkaloid hyoscyamine was made by excised root cultures of *Datura stramonium* (48). Probably the most significant finding from Dawson's work is the very close correlation between the growth rate of the tobacco roots, expressed either as dry wt., length, or number of branches, and the increase in nicotine content (Figure 2) (49). Thus, in this system, growth and the expression of a secondary pathway appear compatible. Subsequent studies showed that roots are the source for natural products ranging from alkaloids to polyacetylenes, flavors, and fragrances (Table II). Because of their slow growth *in vitro*, however, root cultures were displaced and largely forgotten as tools for biochemical studies as fast growing callus and suspension cultures became available in the late 1950's.

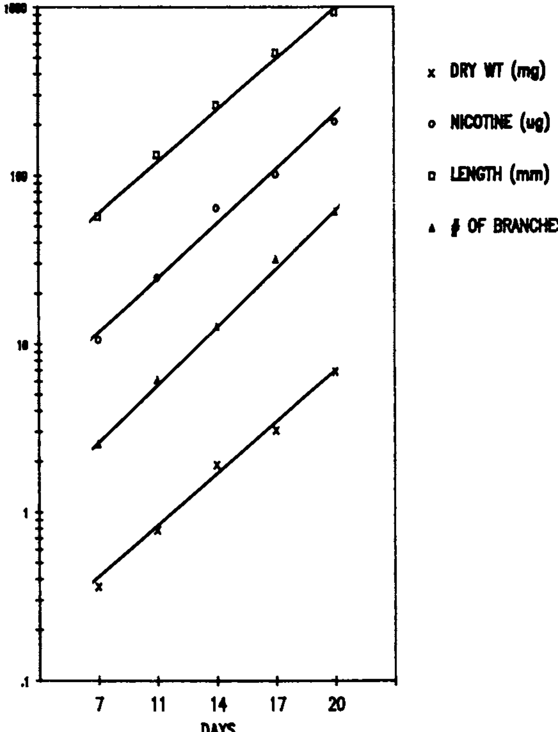

Figure 2. Correlation between growth and alkaloid production in tobacco root cultures. (Reproduced with permission from reference 64. Copyright 1960, American Scientist.)

Table II. Natural Products Synthesized in Plant Roots

Compound	Source	Use	Reference
Hyoscyamine, Scopolamine	*Solanaceae*	Sedatives	(47, 49)
Nicotine	*Nicotiana tabacum*	Insecticide	(48)
Shikonin	*Lithospermum erythrorhizon*	Antiinflamatory	(3, 11)
Rotenone	*Derris elliptica*	Insecticide	(58)
Pellitorine	*Anacyclus pyrethrum*	Insecticide	(58)
Polyacetylenes	*Compositae*	Nematocides	(59)
Sesquiterpene lactones	*Compositae*	Antitumor	(60)
Essential oils	*Santalum album*	Fragrances	(61)
Ginsenoids	*Panax quinquefalium* *Panax ginseng*	Stimulants	(62)
Sanguinarine	*Sanguinaria canadensis*, *Papaver eschscholtzia*	Periodontal disease	(63)

The Use of Transformed Root Cultures

Agrobacterium rhizogenes is the causal agent of the "hairy root" disease affecting a wide range of dicotyledonous species. As in the case of *A. tumefaciens* - induced crown gall, expression of the diseased phenotype involves the stable integration of a portion of the Ri (for root - inducing) plasmid into the plant genome (50). The genes encoded in the Ri T-DNA apparently regulate the balance of endogenous hormones produced by the transformed cell in a way which results in the formation of a fast growing organized system. A similar situation is found in the the tmr (tumor morphology root) mutant of *A. tumefaciens*, which causes the formation of a "rooty" crown gall (51). The "hairy root" character can be transmitted through meiosis in carrot, tobacco and morning glory (52), and the regenerated plants show phenotypic alterations characteristic of the species and/or variety.

Based on the above reports we investigated the possibility of using "hairy root" cultures in studies of secondary metabolism. We focused on the tropane alkaloids, synthesized in the roots of various *Solanaceae* genera (*Atropa*, *Datura*, *Brugmansia*, *Hyoscyamus*, and *Duboisia*). Hyoscyamine and scopolamine are widely used in narcotic medicine as sedatives and in the treatment of sea sickness. Scopolamine also happens to be the best known antidote against nerve gas.

Seeds of *Hyoscyamus muticus* and *H. niger* grown under sterile conditions were infected with the A4 strain of *A. rhizogenes*. Two to three weeks later, the adventitious roots forming at the inoculation sites (Figure 3a) were removed and placed on fresh medium, where excess bacteria were eliminated either by heat or antibiotic (cefotaxime or carbenicillin) treatment. Transformed "hairy root" clones were subsequently established (Figure 3b, c) and maintained in rotary shakers (150 rpm at 30°C; transfer every 3-4 weeks).

Using this protocol about 20 "hairy root" clones of *Hyoscyamus muticus* were established, all of which showed much faster growth than normal root cultures (19). More importantly, "hairy root" clones were shown to produce hyoscyamine (over 80% of extractable alkaloids) at the same levels as in the whole plant or in normal root cultures. The growth and alkaloid yield of at least 3 clones (A4C15, A4C17 and A4C41) have been shown to be stable over at least 20 passages (Figure 4a,b). Transformed tobacco root clones have also been established, showing rapid growth and high nicotine levels (2-4% dry wt). Several additional features of the "hairy root" system make it amenable to the study of secondary metabolism. First, in contrast with the crown gall induced by *A. tumefaciens*, "hairy roots" can be regenerated into whole plants, and the phenotype transmitted through seed (19, 52). Second, "hairy roots" can be readily induced to callus upon transfer to medium containing growth regulators. This unorganized callus produces only trace amounts of alkaloids, but upon transfer back to hormone-free medium it is possible to recover a stable "hairy root" phenotype. This has been shown for at least 4 cycles of root-to-callus transfers, and clearly demonstrates the relationship between an organized state and the expression of a secondary

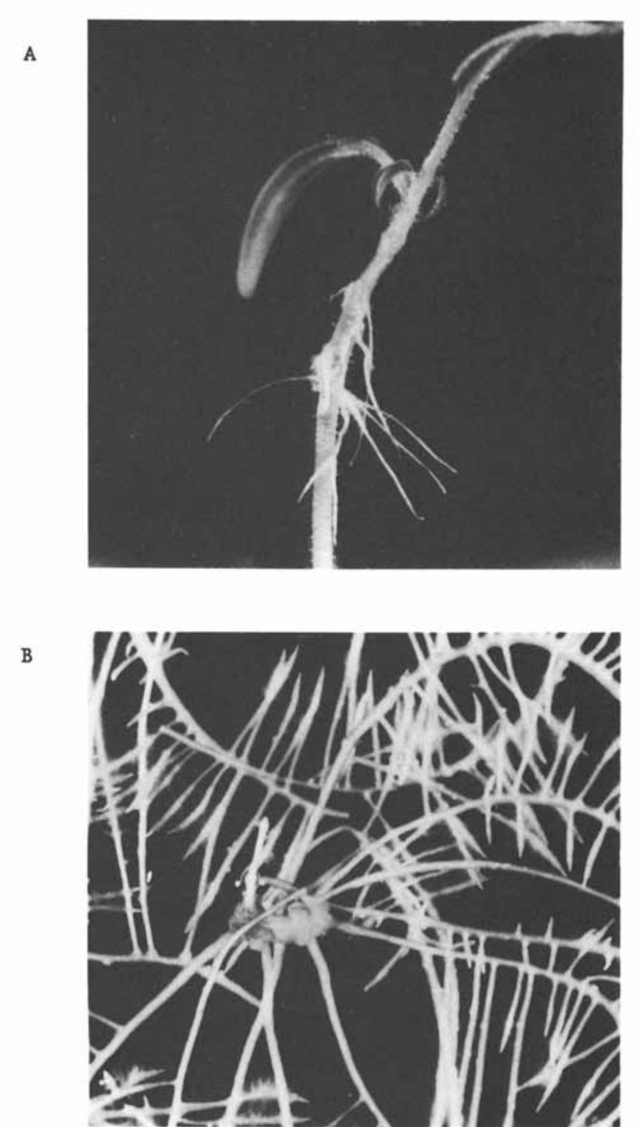

Figure 3. Establishment of "hairy root" cultures. (A) *Datura innoxia* seeding 2 weeks after infection with *A. rhizogenes*. (B) Detail of *Hyoscyamus muticus* "hairy roots".

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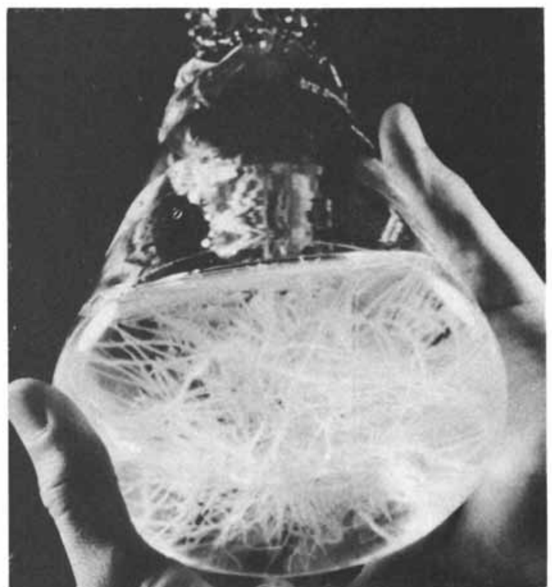


Figure 3. Establishment of "hairy root" cultures. (C) Transformed root clone of H. muticus 4 weeks after transfer.

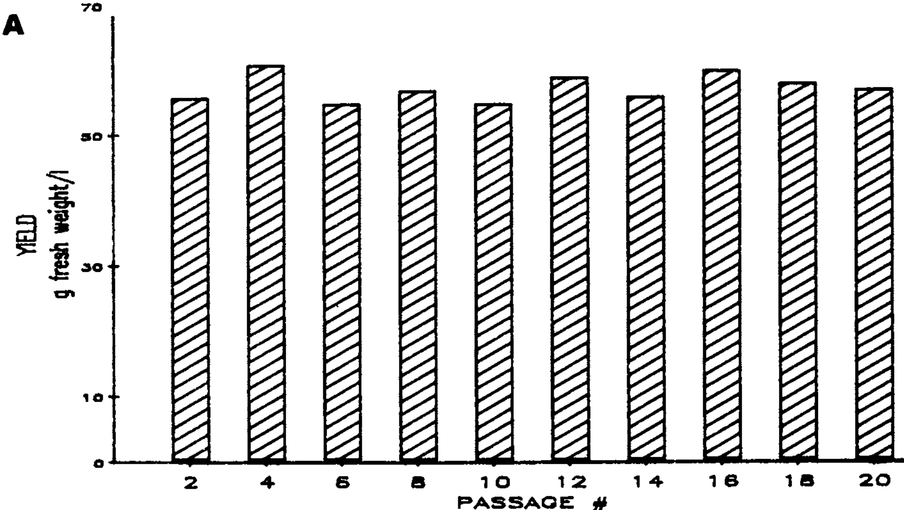
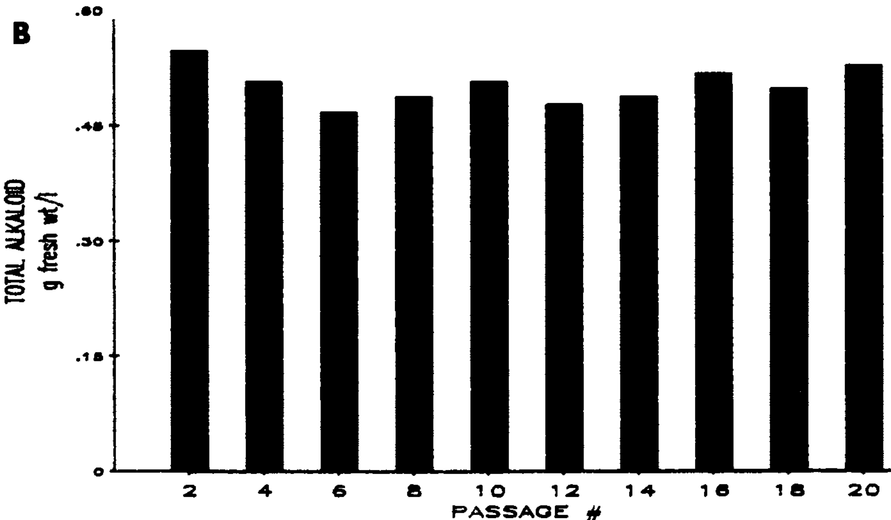


Figure 4. (A) Stability of growth in H. muticus "hairy root" clone A4 cl7.



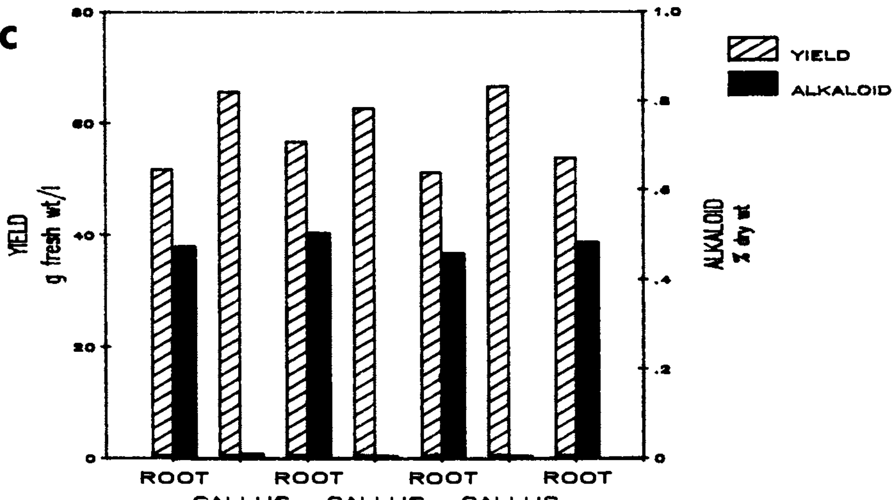


Figure 4. (B) Stability of alkaloid production in H. muticus "hairy root" clone A4 cl7. (C) Stability of "hairy root" phenotype.

metabolic pathway (Figure 4c). Since they can be regenerated at will from callus or suspension cultures, "hairy roots" may provide an ideal system to monitor somaclonal variation. Third, most of the approaches described in the previous sections that lead to accumulation of secondary compounds can be easily applied to "hairy root" cultures. Finally, although work in this respect is only starting, we may expect that scaled-up methods for growth of root clones could be developed in the same way because bioreactors are now available for growth of somatic embryos (53).

Plant roots have been under utilized as a source of natural products. In addition to pharmaceuticals, roots synthesize a wide range of compounds that can be used as natural pesticides (Table II). Renewed interest in allelochemicals will certainly stimulate studies on the biology of compounds made by roots. As mentioned above for the *Nicotiana* alkaloids, it is well established that synthesis of these compounds is closely associated with the metabolism of the rapidly growing and dividing root cells. Therefore, a fast growing, potentially immortal root culture used as an experimental system should contribute useful information to our understanding of secondary metabolism. Recent articles on this research area emphasize the use of organ cultures as the next frontier (13, 16, 54).

Conclusion

The use of plant tissue cultures as sources of natural products is one of the most challenging and promising prospects for what is currently considered plant biotechnology research (21), the other areas being mass cloning through somatic embryogenesis, new variety development through somaclonal variation and gametoclonal variation, and genetic engineering. The ability to transfer genes to alter specific characteristics, and to have them expressed with tissue and temporal specificity is now well established (55). The technology for introduction of plant genes into bacteria and yeast is available, and genetically engineered microorganisms may soon become a source of plant products. The sweet tasting protein thaumatin, found in the leaves of *Thaumatococcus*, has been cloned and expressed in yeast cells (56). However, for secondary metabolic pathways involving the coordinate regulation of many genes, the present challenge lies not in the development of molecular vectors, but in understanding the biology of cell and organ culture systems. Molecular methods will certainly make a powerful contribution to this endeavour, as has already been shown for the pathway leading to phenolics (57). We expect the future breakthroughs in the area of plant secondary metabolism to come from a multidisciplinary approach to the problem. While the systems and strategies outlined in this review reflect some of our biases, their study should provide future insights into the workings of plant cells and organs in culture, so that we may eventually fulfill Alice's dream.

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Chapter 6

Use of Somatic Embryogenesis for the Regeneration of Plants

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Somatic embryos of many crop species can develop in plant cell cultures as the result of discrete manipulations of growth regulators and nitrogen levels in the culture medium. Somatic embryos express some of the same morphological and biochemical attributes *in vitro* as do zygotic embryos, their counterpart *in vivo*. Somatic embryos represent an opportunity to explore the manufacture of embryo specific storage products as well as plant propagules. Techniques for coating naked somatic embryos which will permit survival and subsequent germination are discussed.

In plants, asexual embryo development can be triggered from somatic tissues to occur under a variety of conditions both *in vivo* and *in vitro*. This process is referred to as somatic embryogenesis (1). Somatic embryos are morphologically (2) and biochemically (3,4) similar to their sexually derived counterpart, the zygotic embryo. However, somatic embryos arise by a process of cloning which does not involve meiotic recombination events associated with fertilization and true seed formation. A somatic embryo can be germinated directly into a plant by a one step process similar to seed germination, since it contains both the embryonic shoot and root axes. However, somatic embryos lack structures associated with zygotic embryos such as the seed coat or endosperm. Thus, a somatic embryo develops as a naked embryo.

Somatic embryos can be used for a number of purposes such as for regeneration of genetically modified cells, for production of secondary products and mass cloning for artificial seeds. Figure 1 is a schematic of the process of somatic embryogenesis from explant to regeneration of a cloned plant. Because somatic embryos can be produced from callus cultures (undifferentiated cells), callus can first be subjected to genetic manipulation and somatic cell selection before embryogenesis and recovery of complete plants. Somatic embryogenesis is also an efficient method for recovery of plants

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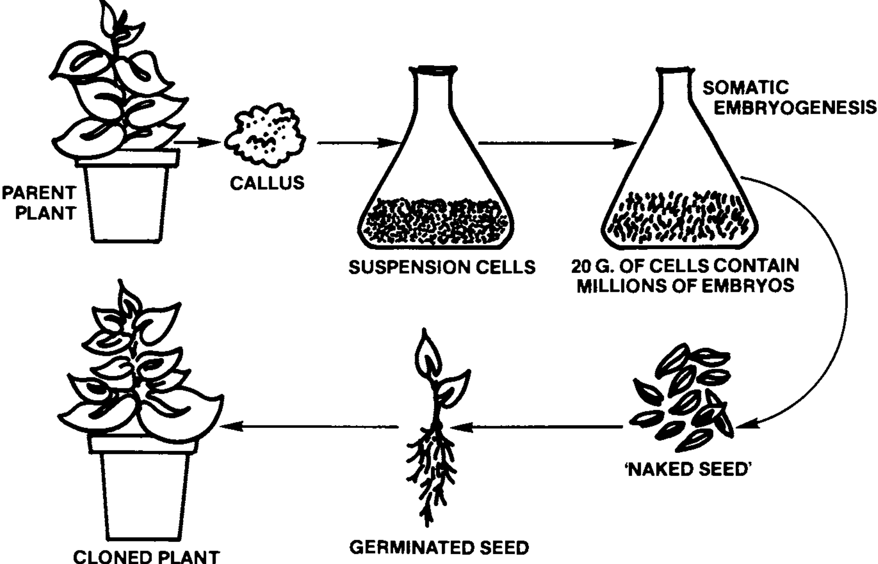

Figure 1. Schematic diagram of the process of somatic embryogenesis.

following experiments employing protoplasts (naked plant cells lacking cell walls) such as electroporation of macromolecules and liposome/cell fusion procedures. Many agronomically important crops such as corn, rice, wheat, soybeans, cotton, and alfalfa can now be regenerated primarily using the process of somatic embryogenesis. Control of this developmental pathway makes many experiments using these species possible.

Because they are biochemically similar to zygotic embryos (3,4), somatic embryos also present an opportunity for in vitro production of numerous secondary plant products which are associated with seeds. For example, essential oil, flavor, and aroma components found in seeds of celery (5), cocoa (6,7), and oil palm (8) are produced by somatic embryos. The ability to control and develop this process may permit more economical and reliable production of certain plant products on an industrial level.

A valuable and more immediate application of somatic embryos is as a cloning tool for plant production. Use of somatic embryos may have an impact on plant breeding and hybrid production. For example, parent inbred lines could be cloned for production of hybrid seed. If these parents contain suitable incompatibility for male sterility, there would be no need to work out restorer systems for these parents, since they could be cloned repeatedly from year to year. Another example for use of somatic embryos is the direct field delivery of hand crossed hybrids where no system of hybrid production exists. In crops such as soybean or lettuce, the pollination biology of the crop does not currently allow for outcrossing of one plant to the next without hand pollinating. If a plant with suitable agronomic traits could be produced, tested, and cloned using somatic embryogenesis, it is conceivable that field delivery of such hybrids would be possible.

Research Problems Associated with Somatic Seeds

Delivery of somatic embryos to a field situation requires that at least three major problems associated with somatic embryogenesis be solved (Figure 2). The first problem is reliable culture control; to differentiate synchronous populations of somatic embryos at a high frequency. Some species such as carrot (9), celery (10), alfalfa (11), coffee (12), and orange (13) have existing, high-frequency protocols available for production of embryos. However, most species have poor somatic embryogenesis systems that produce embryos either sporadically or at much lower frequencies.

A second problem is maturation of the embryo to the stage that it will allow it to germinate autonomously into a vigorous plant in a non-sterile greenhouse or field environment. While procedures exist for normalization of embryo development, tests have not been conducted to assess the field readiness of these embryos, either naked or encapsulated (14,15).

Finally, since somatic embryos are naked, it is presently envisioned that they will need to be coated with a protective covering analoguous to the seed coat of zygotic seeds. The combined covering and somatic embryo is referred to as a somatic seed (Figure 3). The current techniques for production and maturation of somatic embryos requires that this coating be a hydrated gel matrix, but

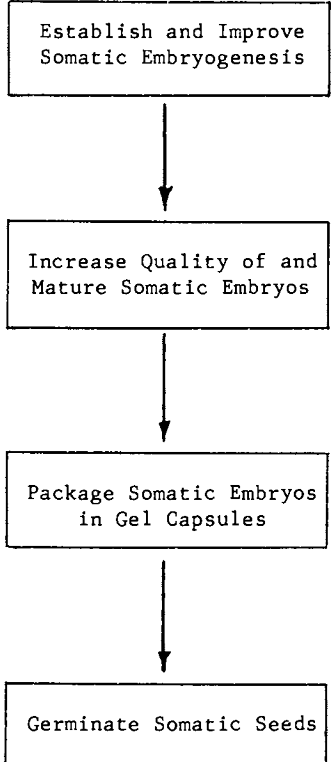

Figure 2. Critical research problems for production of somatic seeds.

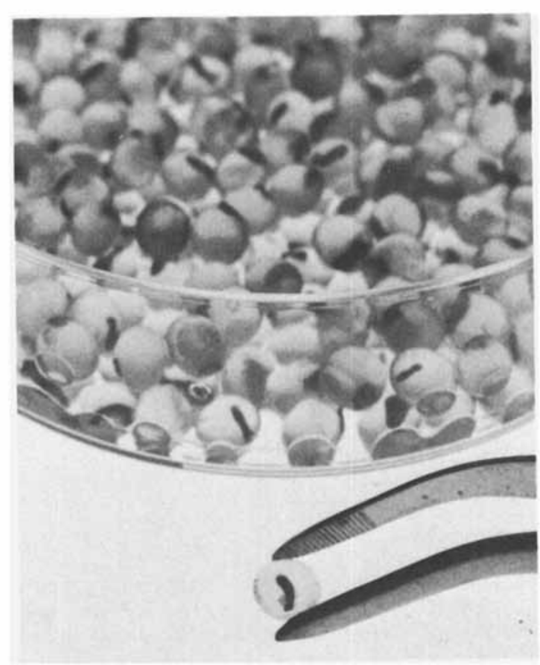

Figure 3. Somatic seeds. Alfalfa somatic embryos encapsulated in calcium alginate beads. Each capsule is approximately 4 mm in diameter.

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there is no reason that a dry coating could not be used if it is compatible with the embryo. As techniques for dehydrating and desiccating somatic embryos develop, dry coatings would be especially useful. Attempts to use dried coatings with somatic embryos of carrot have not given high frequency germination of embryos, however (16,17).

Reliable Culture Control: Improvement in the Frequency of Somatic Embryogenesis in Alfalfa

In alfalfa, and other species, the frequency of somatic embryogenesis can be optimized by adjusting the level of ammonium ion in the regeneration medium (13). The optimal range lies between 10 and 25 mM. In further investigations, the effects of other reduced nitrogen sources such as amino acids have been tested (13). The amino acid which stimulates somatic embryo formation the most is L-proline which causes a 2.5-fold increase in embryogenesis. Other amino acids such as L-glutamine, L-alanine, and L-arginine improve embryo yields compared to the optimized NH_4^+ levels but not to the degree of L-proline. Interestingly, the effect of certain amino acids including L-proline and L-arginine depends on the presence of NH_4^+ in the regeneration medium (19). Thus, ammonium may affect the control of amino acid metabolism in some way as yet not understood.

The frequency of somatic embryogenesis is also effected by optimization of hormone exposure during the induction of embryogenesis and by the genotype used. Earlier work has shown that a three to four day exposure to 50 uM 2,4-dichlorophenoxyacetic acid (2,4-D) and 5 uM kinetin gives the highest yield of embryos (20). Additionally, varieties and lines of alfalfa have been selected and bred for the ability to regenerate (20).

Maturation of the Embryo: Improvements in the Quality of Somatic Embryos in Alfalfa

In addition to increasing the frequency of somatic embryos formation, amino acids also increase the size of the resulting embryos (18). Glutamine, alanine, and arginine give the largest and best formed embryos. Amino acid-treated embryos will also convert more easily to plantlets. Embryos are placed aseptically on dilute culture medium containing no amino acids or hormones. Glutamine-treated embryos give the highest conversion frequency followed by alanine, arginine, proline, and NH_4^+. Thus, treatments which give high frequency do not necessarily result in embryos with the best performance (18). Perhaps combinations of amino acids should be used to achieve a balance between frequency and quality.

Further improvements in embryo quality are achieved by using lower levels of 2,4-D for induction of embryo formation. By treating alfalfa cultures with 10 uM 2,4-D (instead of 50 uM) a 50% increase in embryo conversion was achieved (4). Embryos treated in this manner were morphologically more similar to zygotic embryos than were those produced after a high 2,4-D treatment. Furthermore, the biochemistry of the improved-quality embryos was altered. When the level of embryo-specific 11S storage protein was compared in 10 and 50 uM 2,4-D-induced, a clear difference in protein deposition was evident.

Low 2,4-D treated embryos accumulated 2.4 ug each of the 11S protein found in alfalfa seed, while the 50 uM-treated embryos accumulated less than 20 ng each (Figure 4). This result indicates that the level of storage protein accumulation is related to normalization of embryo development and may represent a useful assay to assess somatic embryo quality (4). The maxiumum level of protein accumulation is less than 10% of the level expected for alfalfa seed, however, suggesting that there is additional room for improvement in somatic embryo quality.

Gel Encapsulation of Somatic Embryos

In order for somatic embryos to be useful as a low-cost, high-volume propagation system, a delivery method is required with the following parameters:

1. The use of low-cost materials that are non-toxic.
2. A provision for singulation of the somatic embryos.
3. The delivery system should be compatible with existing planting equipment.

A hydrogel encapsulation system, particularly one based on alginate, satisfies these requirements. Encapsulation is more advantageous than fluid drilling because it singulates the embryos and allows for delivery using existing vacuum or cup planters. As discussed, the quality of the somatic embryos is a further critical parameter for producing artificial seeds. Improvement in both the quality of embryos and the gel encapsulation technology need to progress simultaneously.

Somatic embryos of four species, alfalfa, celery, *Brassica*, and lettuce were encapsulated in a variety of hydrogels (21,23; Figure 3). The most useful hydrogels in terms of ease of encapsulation and lack of damage to the somatic embryos are sodium or potassium alginate, sodium alginate with gelatin, and carrageenan with locust bean gum. In general, the somatic embryos were mixed with the uncomplexed hydrogel and dropped into an appropriate complexing reagent. Capsule hardness, as measured with a Chatillon push-pull gauge, range from 0.2 to 1.2 kg breaking pressure per capsule. This range of hardness is sufficiently firm for handling the capsules, but does not impede somatic embryo germination. Germination of somatic embryos from the capsules of these gels is equal to non-encapsulated somatic embryos.

Sodium and potassium alginates are essentially equivalent in ability to form capsules. Consequently, sodium alginate was chosen due to its availability and lower cost. Sodium alginate forms suitable capsules at concentrations ranging from 0.5 to 5.0% (w/v) when dropped into either a calcium chloride or calcium nitrate solution (30 to 100 mM range). Other complexing agents such as lanthanum chloride, cobaltous chloride, or ferrous chloride are also suitable for complexing alginate. Complexation time is 20-30 minutes. Sodium alginate (2-3% w/v) and calcium chloride (100 mM) are the standard components for encapsulating somatic embryos in the great majority of our experiments.

Sodium alginate (2% (w/v) when mixed with gelatin (5% w/v) produces softer capsules that still retained sufficient integrity for

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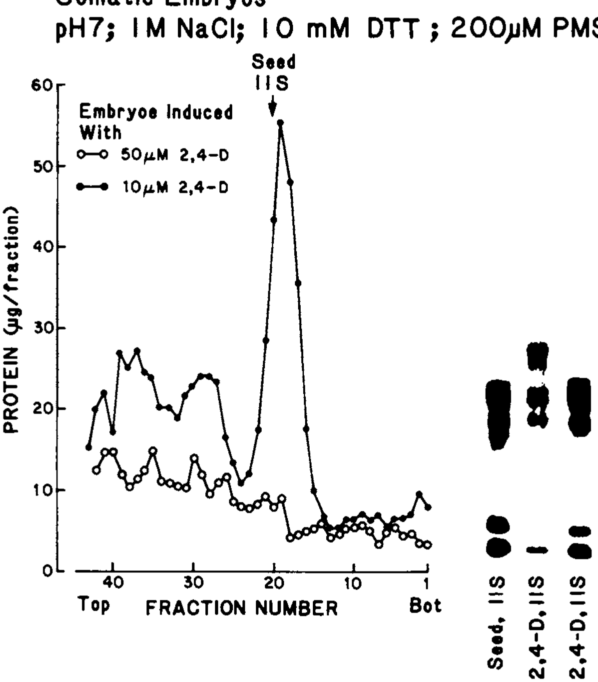

Figure 4. Alfalfa somatic embryo proteins separated after extraction by sucrose density gradient separation (left side of chromatogram) and SDS-polyacrylamide electrophoresis (right side of chromatogram). The 2,4-D-treated embryos express more 11S protein as shown in the sucrose gradient. The 11S protein has polypeptides that co-migrate with 11S seed protein as shown by SDS-PAGE. (Reproduced with permission from reference 4. Copyright 1985, Plenum Publishing.)

handling. The sodium alginate-gelatin mixture is held at 40°C prior to complexation in a 50 mM solution of calcium chloride. This temperature does damage the embryos. The mixture may be useful for encapsulating somatic embryos that do not germinate vigorously and will not emerge from a harder capsule.

Carrageenan (0.2 to 0.8% w/v) and locust bean gum (0.4 to 1.0% w/v) produce firm capsules when dropped into a 500 mM solution of either potassium chloride or ammonium chloride. These capsules are similar in hardness to the alginate-gelatin capsules. In order to produce firm capsules, a carrageenan that was rich in kappa-carrageenan is required. Locust bean gum is essential for encapsulating somatic embryos since it lowers the gelling temperature of carrageenan from 50°C (detrimental to somatic embryos) to 30°C.

Much of the embryo encapsulation research has been conducted using somatic embryos of either alfalfa or celery. Initial results indicate that for albuminous species with a prominent endosperm such as celery, additional carbohydrate storage reserves are required in the capsule for germination, whereas for ex-albuminous species with reduced endosperm additional carbohydrates are not necessarily required (23). It appears that the capsule itself may serve as an artificial endosperm tailored to the requirements of species of concern.

Finally, germination of the somatic seeds and conversion to complete, vigorous plants is essential. To date, research has focused on *in vitro* conversion experiments with celery and alfalfa. For both crops, the conversion frequencies for somatic seeds is identical to that for naked somatic embryos. For celery, this is 60-90% while alfalfa conversion is 40-50%. Although the frequencies vary from experiment to experiment, within any one experiment, somatic seeds and naked embryos show the same response.

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Chapter 7

Selection for Herbicide Resistance at the Whole-Plant Level

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We have isolated single, nuclear, dominant mutations in whole plants of the small crucifer *Arabidopsis thaliana* which confer a high level of resistance to a sulfonylurea herbicide. The ease and rapidity with which such mutations can be isolated using *A.thaliana* suggests that the approach may be of broad utility for studies of herbicide resistance and mode of action.

The growing dependence of modern agriculture on herbicidal compounds has led to the development of hundreds of commercial herbicides which inhibit a wide variety of plant processes (1). By analogy with bacterial antibiotics, the primary target of many herbicides is a specific step in a biochemical or physiological pathway and may involve only a single protein (2,3). It is not surprising, therefore, that single mutations in genes encoding such proteins, can increase the resistance of a plant to a herbicide (2,4-7). These mutations may cause a loss of function by decreasing herbicide uptake or decreasing the activity of an enzyme that normally metabolizes the herbicide to a toxic form. Herbicide resistance can also occur by a gain-of-function mutation which increases catabolism of the herbicide to a nontoxic form, overproduces a component affected by the herbicide, or alters the structure of a protein thereby decreasing protein-herbicide interaction.

Herbicide-resistant plant varieties have proven to be valuable experimental tools in determining the molecular mode of action of herbicides (2,8). In addition, such varieties are likely to be an important source of selectable markers for use in plant molecular genetics and in the engineering of resistant crop species (9). The earliest herbicide-resistant biotypes described arose spontaneously from weed populations which had been repeatedly exposed to a herbicide (10,11). More recently, mutagenesis and selection on defined media have been used to isolate herbicide-resistant or herbicide-tolerant mutants of higher plants from populations of cells in tissue culture (4,5,7,12). While the use of plant tissue culture has proven useful for isolation of some types of mutants, the

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technique has several drawbacks: [1] regeneration of the mutants may be difficult or impossible, [2] mutations expressed in culture may be unstable or may not be expressed at the whole plant level, [3] some herbicides may not be lethal at the cell culture level, and [4] the time-scale for mutant isolation and plant regeneration can be relatively long.

Although direct selection at the whole plant level has been successfully used to isolate auxin-resistant mutants of *Arabidopsis thaliana* (*6*), the approach has not been widely used. We describe here the selection at the whole plant level of rare mutants of *A. thaliana* resistant to the herbicide chlorsulfuron. We suggest that the approach described here can be easily and inexpensively applied to the isolation of mutants resistant to any one of a wide range of herbicidal compounds. After producing M2 seed it requires only a few hours of work to carry out the manual manipulations and the result is available in less than two weeks. The selection is rapid, simple, requires relatively little space and has none of the drawbacks associated with tissue culture. In principle, the technique can be easily applied to any plant which is easily mutagenized and has a small seed size. However, the use of *A. thaliana* with its small genome, short life cycle, ease of handling and well-defined genetics (for recent reviews see *13,14,15*) increases the probability of isolating a specific mutation and facilitates gene isolation and subsequent genetic and molecular analysis.

Chlorsulfuron

Chlorsulfuron (2-chloro-N[(4-methoxy-6-methyl-1,3,5-triazin-2-yl) aminocarbonyl]benzenesulfonamide) is a potent sulfonylurea herbicide produced by DuPont. Several lines of evidence strongly support the idea that sulfonylurea herbicides act by specifically inhibiting acetolactate synthase (ALS), the first enzyme of the branched-chain amino acid biosynthetic pathway (Fig-1). First, effects of the herbicides are reversed by exogenously supplied isoleucine and valine, the end products of the branched-chain amino acid biosynthetic pathway (*16-18*). Second, ALS activity is inhibited *in vitro* by low concentrations of the sulfonylurea herbicides (*8,16,18,19*). Finally, sulfonylurea-resistant mutants of *Escherichia coli*, (*20*), *Salmonella typhimurium* (*16*), yeast (*19*) and tobacco (*4*) have been isolated. In each case the dominant herbicide resistant phenotype cosegregates with a sulfonylurea-resistant ALS activity. The mutations conferring resistance in enteric bacteria and yeast are known to map to the ALS structural genes *ilvG* and ILV2, respectively (*16,19,20*).

A. thaliana is rapidly killed by low concentrations of chlorsulfuron. When seeds were placed on a defined agar medium (*21*) containing concentrations of herbicide exceeding 28 nM (10 ppb), seedling emergence was completely inhibited. As expected, addition of 1.0 mM isoleucine and valine together, but not separately, allowed germination even in the presence of 280 nM chlorsulfuron. Thus, it appears that the only target in *A. thaliana* for the herbicide is branched-chain amino acid biosynthesis. Although it is convenient to know beforehand that there is a single mode of toxicity, it is not a prerequisite to the isolation of resistant mutants.

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Mutagenesis

Mutagenesis is used to increase the mutation frequency in *A. thaliana* over 1000-fold (22), thereby making feasible the isolation of very rare mutants. Although a wide range of radiation and chemical treatments are effective in mutagenizing *A. thaliana*, a simple procedure involving the exposure of seed to the mutagen ethyl methane sulfonate (EMS) is commonly used (21). This compound is believed to cause mutations by alkylating the N-7 or the O-6 positions of guanine (23). The fate of N-7 alkylated guanine is uncertain. However the O-6 methylation appears to cause primarily GC to AT transitions. This possibility of specificity implies that not all possible mutations will be represented in an EMS-mutagenized population irrespective of the population size. Thus, the use of another mutagen with different specificity may be advisable under some circumstances.

Mutagenized seed of *A. thaliana* gives rise to M1 plants chimeric for a large number of heterozygous mutations. Spontaneous self-fertilization of M1 plants produces M2 seed carrying those mutations induced in one of the two embryonic germline cells (24). The M2 rather than the M1 generation is used for mutant isolation because M2 plants are no longer chimeras and nuclear mutations have segregated to produce homozygous seed.

Redei (25) has calculated the necessary size of the M1 population and the number of bulked M2 plants that need to be screened in order to have a high probability of recovering a recessive loss-of-function mutation. Although a recessive loss-of-function mutation can result from any one of a number of nucleotide substitutions, additions or deletions within a specific gene, a dominant gain-of-function mutation is likely to be caused by only one or a few changes within the genome. For example, varietal resistance to Atrazine appears to be conferred by a relatively few specific changes within the gene for the 32kd protein (26,27). Because many mutations causing herbicide resistance are a result of relatively rare gain-of-function mutations, it is important to determine the size of the M1 and M2 populations needed to recover any mutation within the genome. Under our standard conditions for EMS mutagenesis, the frequency of recovery of simple loss-of-function mutants for any one of several different enzymatic activities is approximately 1 per 2000 M2 plants (28-30). Using this number as an estimate of the average frequency of loss-of-function mutants and some broad assumptions, we can calculate the probability of saturating the genome with mutations under our standard mutagenesis conditions. To do this we first need to estimate the number of different EMS mutational events which can give rise to a loss-of-function mutant. The percentage of GC to AT transitions which result in an amino acid substitution or chain termination is 60.4% and 5.2%, respectively. If we assume that there is no bias in codon usage, then 65.6 represents the maximum percentage of EMS induced changes which could result in a loss of function. However, many amino acid substitutions have a neutral effect on the function of a protein. It has been estimated (31) that

only 42% of amino acid substitutions affect the lac repressor. Using the lac repressor as a model it can be estimated that (42% x 60.4%) + 5.2% or 31% of EMS-induced changes result in a loss-of-function.

If we assume that on average the coding region of a gene is 1500 bp and that the GC content of the *A. thaliana* chromosome is 41.4% (32), then the possible number of EMS induced changes per gene is 1500 x 0.414 or 621. A given loss of function mutant could have any one of 31% x 621 = 193 different mutations. Therefore, the frequency of inducing a mutation in a specific nucleotide is 1/[2000 x 193] = 1 mutation in 386,000 M2 plants. With this number as an estimate, the size of an M2 population required to represent every possible EMS-induced homozygous mutation (excepting gamete or embryonic lethals) with a given probability can be calculated using the binomial equation:

$$N = \frac{\ln(1 - P)}{\ln(1 - X)}$$

Where P = probability that every mutation is represented, N = the number of plants in the population and X = the frequency of occurrence of a specific mutation in the population. Using the values derived above and a probability of 95%, this gives a value of:

$$N = \frac{\ln([1-.95]}{\ln[1-1/386000]}$$

Where N = 9.99×10^5 plants and it is assumed that the M1 population size is not limiting. The number of viable fertile M1 plants that are needed to generate the necessary M2 diversity is then calculated by the formula:

$$\frac{\text{\# M2 plants needed to cover all mutations}}{\text{ploidy factor x GECN}}$$

In *A. thaliana* the genetically effective cell number (GECN) is two (24) and the ploidy factor is four (a mutation heterozygous in the M1 parent has a 25% chance of being homozygous in a given M2 progeny). Thus, approximately $9.99 \times 10^5/8$ = 125,000 M1 plants are needed.

Our M1 populations are, generally 100,000 to 200,000 plants in size. Thus, the calculations above suggest that we routinely saturate the genome with all possible EMS-induced mutations. Furthermore, the high seed yield per plant and the small size of *A. thaliana* seed make mutant selections using 1.0×10^6 M2 seed feasible.

One additional calculation can be derived from the discussion above. The size of the *A. thaliana* genome is 70,000 kb (32). The total number of possible EMS-induced changes is $7 \times 10^7 \times 0.414 = 2.9 \times 10^7$. Thus, if any given mutation occurs at a frequency of 1/386000 plants then there must be 2.9×10^7 homozygous mutations per 3.86×10^5 plants or 73 homozygous mutations per M2 plant; 219 including heterozygous mutations. Many of these mutations will not affect the function of any gene because they cause a neutral change or are not within the coding region of a gene. Nevertheless, such a calculation

underscores the need to decrease the number of background mutations in a newly isolated mutant by repeated backcrossing to a wild type strain.

Selection of Herbicide Resistant Mutants

Herbicide resistant mutants are selected on a solid sterile selection medium prepared by autoclaving a minimal salts nutrient solution (21) with 0.7% agar. After cooling to 50C the herbicide is added and the medium is distributed to 9 cm petri plates. Initially a range of herbicide concentrations should be tested to determine the lowest concentration of herbicide that kills germinating wild-type seedlings effectively. This concentration is then used for the large-scale selection of M2 seed.

Seed is sterilized for 15 minutes in a solution containing 5% sodium hypochlorite (30% laundry bleach) and 0.02% Triton-X100 at a concentration of 1000 seed/ml. Following treatment, seeds are rinsed 5 to 10 times with sterile distilled water. Large numbers of sterilized seed can be distributed onto plates by rotating plates on a bacteriological turntable and delivering a spiral of seed onto the surface of the spinning plate from a pasteur pipet (Fig. 2). A piece of cheesecloth placed on the surface of the agar prevents clumping of the seed. Using this technique we have distributed up to 10,000 M2 seed onto a single petri plate. Such a high plating density is only practical for selection purposes if the herbicide kills at a very early stage of development, as is the case for chlorsulfuron. By contrast, *A. thaliana* seedlings develop past the cotyledon stage on even high (1 mM) concentrations of glyphosate (Haughn and Moffatt, unpublished). At plating densities higher than 2000 seed/plate, mutants resistant to glyphosate would not be easily distinguished from sensitive seedlings due to loss of visual resolution.

Selection plates with M2 seed are incubated at 21 to 25C under continuous fluorescent illumination at about 80 uE m^{-2} s^{-1}. Herbicide resistant mutants should be easily recognizable after 5 to 10 days. Putative resistant plants are transplanted to soil and retested for resistance in the subsequent generations.

Chlorsulfuron Resistant Mutants

Three hundred thousand *A. thaliana* M2 seed were placed on 30 petri plates containing 0.2 uM (75 ppb) chlorsulfuron. Four putative chlorsulfuron resistant mutants were readily identified against a background of non-germinated seeds. When progeny from these plants were retested in the M3 generation, three of the four lines proved to be resistant to chlorsulfuron. One of the mutant lines, designated GH50, was tested in the M4 generation for its level of resistance. Six day old seedlings were transferred to plates containing a range of concentrations of the herbicide and incubated at 24C in continuous illumination for an additional two weeks. This line showed a level of resistance 300 to 1000-fold higher than that of the wild type (Fig. 3).

Genetic analysis has shown that of the three resistant plants

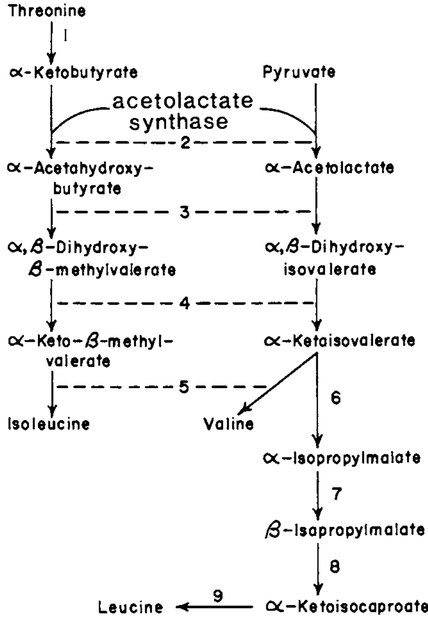

Figure 1. The branched-chain amino acid biosynthetic pathways of enteric bacteria (35). The biosynthetic step catalyzed by acetolactate synthase is step 2.

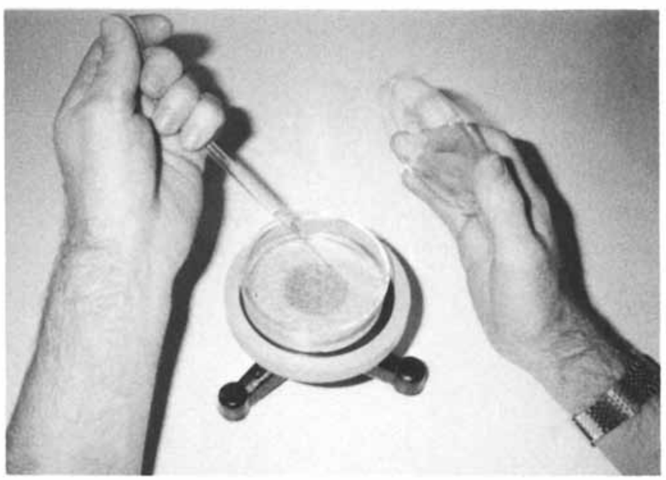

Figure 2. Illustration of the technique used to distribute high numbers of *A. thaliana* seed onto petri plates.

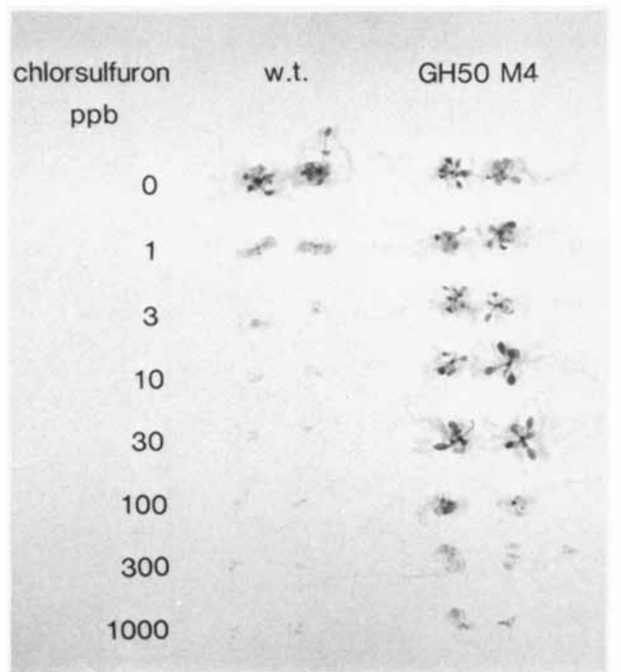

Figure 3. The effect of increasing concentrations of chlorsulfuron on the growth of wild type and resistant (GH50) lines of *A. thaliana*.

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originally selected, one was heterozygous and two were homozygous for a single nuclear mutation at a locus we have designated *csr* (Haughn and Somerville, in preparation). Thus the frequency of a homozygous mutant in the M2 population was no higher than 1 in 1.5×10^5, or approximately 75-fold lower than the frequency of a simple loss-of-function mutation. If 193 EMS-induced mutations/gene can give a loss of function (see section on mutagenesis), then it is likely that only 1 to 3 nucleotides/genome can be altered by EMS to give resistance to 0.2 uM chlorsulfuron. This result is expected of a dominant gain-of-function mutation.

The frequency of the chlorsulfuron-resistance mutations has some practical implications with respect to the frequency with which herbicide-resistant weed biotypes may be expected to arise following agricultural use of the herbicide. Our estimate is that a spontaneous mutant comparable to those described here should arise at a frequency of about 1 per 10^9 plants (ie., 1 per 150000 for a mutagenized population in which the frequency was increased 5000-fold). If we assume that a typical number of viable weed seeds per hectare is about 0.13 billion (33) then we may expect to find about 1 chlorsulfuron-resistant weed per 8 hectares.

Our preliminary results indicate that, like the chlorsulfuron-resistant tobacco mutant, the *A. thaliana* line GH50 has chlorsulfuron-resistant ALS activity *in vitro* (34). It is possible, therefore, that the chlorsulfuron-resistance mutation of GH50 lies within the structural gene for ALS. In the absence of chlorsulfuron the mutants do not grow as quickly as the wild-type. However, it is not yet clear whether this is related to the herbicide resistance or to the many other mutations which are induced during mutagenesis.

Discussion

We have described methods which allow the selection of relatively rare chlorsulfuron-resistant mutants in *A. thaliana* at the whole plant level. We suggest that the approach is of broad utility for the investigation of herbicide mode of action because of simplicity and speed. The approach is so simple that it can be used as a diagnostic tool to determine the relative frequency with which spontaneous mutants resistant to a given herbicide arise - an important criterion in determining the usefulness of a newly developed herbicide.

Assuming that the chlorsulfuron resistance in the *A. thaliana* mutants described here is due to a mutation within the ALS structural locus, it may be possible to clone the resistant allele. Such a resistance gene should provide a very useful selectable marker for plant transformation studies. Indeed, transfer of the gene to other species may be useful in extending the agronomic utility of the herbicide.

The approach described here is not restricted to sulfonylureas. Our laboratory has successfully applied these techniques in isolating mutants resistant to the herbicides 2,4-dichlorophenoxyacetic acid (Estelle and Somerville, in preparation) and an Imazidolinone

herbicide (Haughn and Somerville, in preparation). By contrast, we have not yet been able to identify mutations which confer high-level resistance to glyphosate. This is particularly intriguing in view of the fact that single gene resistance to this herbicide has been established in microorganisms. It is possible that in the case of glyphosate resistance in *A. thaliana* the required amino acid substitution cannot occur by way of a GC to AT transition.

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Chapter 8

Classical Approaches to the Development of Herbicide Tolerance in Crop Cultivars

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Development of herbicide tolerant crop cultivars may be an alternative approach to weed control. Classical breeding methods have met some success in developing herbicide tolerant cultivars including metribuzin tolerant soybean and triazine tolerant canola varieties. Classical breeding has relied primarily on intraspecific genetic variation from sexual hybridization/recombination/segregation and to a lesser extent, mutation-induced variability. The intraspecific hybridization approach to herbicide tolerance has been limited by lack of "available" intraspecific variability, complex inheritance (multigenic) and low heritabilities associated with tolerance to several specific herbicides. Complex inheritance and low heritabilities may also be responsible for limited progress from mutational breeding. Utilization of vast interspecific differences in resistance to most herbicides is beyond the capacity of classical breeding methodology which requires a degree of sexual compatibility between potential sources and targets of herbicide tolerance. Emerging technologies including *in vitro* mutagenesis and/or selection, parasexual hybridizations, embryo rescue, and ultimately genetic transformation should impact development of herbicide tolerant crop cultivars. These technologies will not circumvent classical breeding methodology due to inherent deficiencies, but should provide desirable sources of heritable herbicide tolerance that can be recombined, refined, multiplied, and distributed through classical variety development technologies.

While breeding objectives and approaches vary widely with species, most plant breeding programs are concerned primarily with exerting

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selection pressure on genetically variable populations. Selection pressure (selective inclusion of desirable or exclusion of undesirable plants from the population) is commonly based on performance, stability and/or quality parameters. Genetically variable breeding populations are usually generated through artificial or natural hybridizations between complementary parents, recombination and segregation; or through mutagenesis in combination with hybridization and/or recombination and segregation. Breeders thus apply selection pressure to breeding populations (usually for several desired or required characteristics) to identify or increase the frequency of superior genotypes that arise through random assortment of genes following recombination and segregation or through induced mutations.

Classical breeding may be limited by a lack of adequate genetic variability or lack of an effective selection method for a desired characteristic. Inadequate availability of genetic variability is a major limitation in developing novel herbicide tolerant varieties. Since classical breeding relies on the sexual cycle for recombination of genes, sources of desirable genetic variability for herbicide tolerance are restricted to other plants with at least partial sexual compatibility with the species or crop of concern. Genetic variability for tolerance to many herbicides, including triazines is available within most crop species, but is often very limited (1,8), complex and commonly characterized by low heritability (14,15). When major simply inherited genes for herbicide tolerance are identified, as for metribuzin tolerance in soybean (3), such genes can be utilized by classical methods to develop tolerant varieties without major difficulty.

Although vast differences in tolerance to most herbicides occurs between plant species, major genes controlling herbicide resistance are extremely rare within the available germplasm of several normally susceptible economic species, in spite of systematic screening of catalogued germplasm in some cases (R.G. Palmer pers. comm.). When variability for a desired characteristic is not available, the classical breeding options are limited to mutagenesis or introgression (interspecific transfers). Success in the isolation of herbicide (including triazine) tolerance among plant cells following _in vitro_ mutagenesis and/or selection (6,11) suggests that _in vivo_ mutagenesis could be effective for induction of desirable genetic herbicide tolerance. _In vivo_ mutagenesis-selection schemes, however, have not made a major contribution in the development of herbicide tolerant crop varieties to date. Similarly, other classical mutation approaches including plastome mutators, which increase the frequency of plastid mutation (2,13), although potentially useful, are not readily applicable in most crop species, and thus have not played an active role in the development of herbicide tolerant varieties to date.

Cytoplasmic triazine tolerance.

Since a number of triazine tolerant weed biotypes have been discovered through triazine application to crop lands, development of

herbicide tolerant varieties has been possible through introgression. Breeding, physiological and molecular genetic studies (2,4,5,7) have indicated that triazine tolerance in several weed biotypes is cytoplasmically inherited. The tolerance results from a single amino acid alteration in the triazine receptor protein. This protein, involved in electron transport, is associated with Photosystem II and is coded for by photogene 32 of the chloroplast chromosome. Triazine tolerant biotypes commonly have reduced photosynthetic rates (2,5) and somewhat reduced vigor.

Development of tolerant oilseed rape varieties.

Identification of a triazine tolerant biotype of *B. campestris* L. (a.k.a. wild turnip, birds rape, *B. rapa* L.) has allowed the development of triazine tolerant *B. napus* L. oilseed rape cultivars and tolerant rutabaga genotypes. Development of the triazine tolerant *B. napus* varieties was accomplished through introgression of the tolerant weed biotype cytoplasm into *B. napus*. The transfer was accomplished through a combination of backcrossing and cytogenetic selection (Figure 1), in which the triazine tolerant weed biotype served as the donor and a susceptible canola variety served as the recurrent pollen parent (4). The transfer was completed and stabilized by the second backcross, although additional backcrosses were required to eliminate undesirable high levels of erucic acid and glucosinolates. Stabilized backcross progeny of this material provided a triazine tolerant *B. napus* donor for Dr. Souza-Machado and collegues to continue the transfer of tolerance into rutabaga (also *B. napus*), again through backcrossing.

Transfer of the tolerant birds rape cytoplasm to *B. napus* has resulted in stable, highly tolerant genotypes of oilseed rape, one of which has been released as a canola variety, OAC Triton, with another variety, selected in Saskatchewan (tentatively named Tribute) expected for release later this year.

Performance of triazine tolerant varieties.

In comparison to recurrent susceptible counterparts the performance of OAC Triton and a series of other triazine tolerant backcross derivatives of oilseed rape varieties has been somewhat disappointing (Figure 2). A more recent study designed to precisely compare the susceptible and triazine tolerant cytoplasms in identical nuclear backgrounds using reciprocal single cross hybrids clearly demonstrated significant yield depression as a result of the cytoplasm (Table 1). Although selection pressure in triazine tolerant breeding populations is improving agronomic performance of tolerant oilseed rape, there is and may continue to be a cost associated with the herbicide tolerance extracted from the birds rape cytoplasm.

Producer acceptance.

Producers appear to be accepting triazine tolerant varieties and triazine herbicides as a reasonable method of controlling weeds

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BRASSICA CAMPESTRIS (BIRDSRAPE MUSTARD) TRIAZINE TOLERANT WEED ♀ X BRASSICA NAPUS (OILSEED RAPE) TRIAZINE SUSCEPTIBLE CROP ♂

F1 HYBRID TRIAZINE TOLERANT X B. NAPUS (OILSEED RAPE) (RECURRENT POLLEN PARENT)

BACKCROSS 1 X B. NAPUS (OILSEED RAPE)

MORE BACKCROSSES TO B. NAPUS

TRIAZINE TOLERANT OILSEED RAPE

Figure 1. Transfer of the triazine tolerant cytoplasm from a weed biotype to oilseed rape by backcrossing.

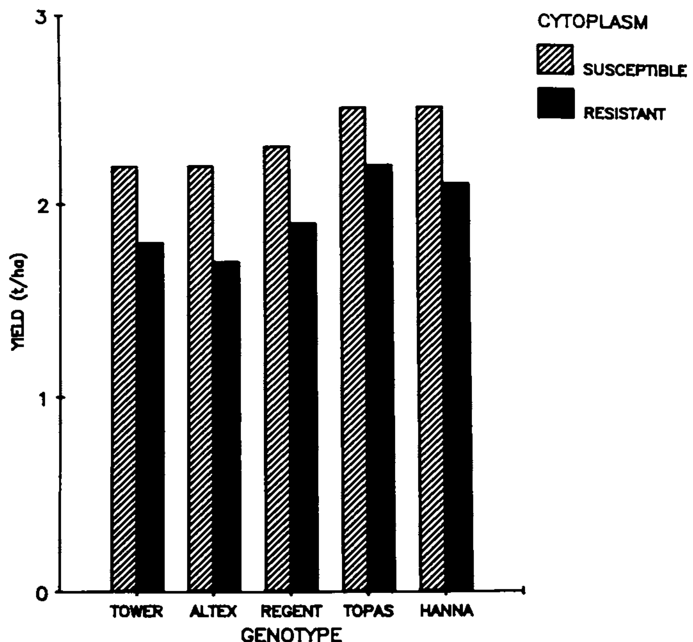

Figure 2. Comparative yield performance of susceptible varieties and their triazine tolerant backcross five derivatives.

Table 1. Comparative agronomic performance of triazine tolerant and susceptible cytoplasms in identical nuclear backgrounds (reciprocal single cross hybrids) of oilseed rape

Genotypes	Yield (t/ha)	Flowering (dap)[1]	Maturity (dap)	Lodging (1-5)[2]	Height (cm)
'OAC Triton'	2.3 d[3]	51 ab	94 a	3.3 bc	112 a
'Topas'	2.6 bcd	53 bc	96 a	1.0 a	126 bc
'Hanna'	2.8 bc	55 cd	96 a	1.4 ab	127 bc
Triton x Topas[4]	2.2 d	56 cd	95 a	1.0 a	124 b
Topas x Triton	3.0 ab	55 cd	95 a	1.0 a	131 bc
Triton x Hanna	2.4 cd	56 cd	95 a	2.1 bc	114 a
Hanna x Triton	3.3 a	54 bce	96 a	1.5 ab	137 c

1 Days after planting.
2 Linear scale from erect (1) to prostrate (5).
3 Numbers within columns not followed by at least one common letter are different at the $p \leq .05$ level according to Duncan's new multiple range test.
4 Female parent (cytoplasmic donor) is designated first.

commonly associated with canola production in Canada in spite of the performance deficit associated with triazine tolerant oilseed rape varieties. This is probably due in part to the lack of alternative herbicides for controlling some of the most serious weeds in traditional canola production areas. Similar economic/production/crop management can be expected to play a role in sorting out the value of other herbicide tolerant crop cultivars, regardless of sources.

Other potential transfers by classical breeding.

Most of the triazine tolerant weed biotypes identified to date have economic relatives but few if any are as closely related to economic crops as the birds rape. To date, this cytoplasm has been transfered to oilseed rape, rutabaga and oriental mustard by several breeding groups. Partial cross-compatibility with several other *Brassica* species including the cole crops, and other more distantly related species (eg. *Diplotaxis* and *Raphanus* species) may permit transfer by classical methods (perhaps assisted by embryo rescue techniques). Chloroplast or mitochondrial X nuclear incompatibilities however, may limit this potential. As other triazine tolerant weed biotypes emerge, opportunities for transfer to closely related economic species by classical methods may evolve.

Emerging biotechnologies.

Embryo rescue technology, protoplast or cytoplast by protoplast fusions (9,12), and various DNA transformation techniques (10) are gradually breaking the sexual barriers that restrict transfers via classical hybridization. These technologies along with *in vitro* mutagenesis and selection should have a very positive impact on variety development, particularly as it relates to development of herbicide tolerant varieties in the foreseeable future. Such technologies will not diminish the need for classical breeding methods as the number and turn-over rate of crop cultivars precludes common use of these complex, unpredictable and expensive technologies for direct cultivar development; but may provide sources of heritable herbicide tolerance traits for breeders to incorporate into adapted crop cultivars.

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Chapter 9

Cell Culture Approaches for Obtaining Herbicide-Resistant Chloroplasts in Crop Plants

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Genetic techniques for obtaining chloroplast-encoded herbicide resistance in crop plants are reviewed. These are based on the use of cell culture methods and include mutant selection in cultured cells, chloroplast transfer via protoplast fusion, and genetic recombination between chloroplast genomes. The first two methods are described in detail using triazine resistance as an example.

The characterization of triazine-resistant weed biotypes as chloroplast mutants (1,2) generated interest in the possibility of obtaining crop plants carrying herbicide-resistant chloroplasts. It has been possible to introduce herbicide resistance into crops that have closely related resistant weed species by crossing. One example is *Brassica napus* (oilseed rape), for which *B. kaber* and *B. campestris* are problem weeds (3). Herbicide resistance would also be of value in crop species where herbicide carryover is a problem. One example is the rotation of corn, a triazine tolerant crop plant, with soybean, which is sensitive to these herbicides (1). If triazine-resistant soybean varieties were available the problems posed by herbicide carryover could be eliminated. Chloroplast genes conferring herbicide resistance have an advantage over nuclear-encoded genes. Nuclear genes can be transferred into related interfertile weeds via pollen. However, chloroplast genes are maternally inherited. For this reason the chloroplast-encoded herbicide resistance cannot become established in the weed population via cross-pollination (3).

Cell culture-based methods suitable for obtaining chloroplast-encoded herbicide resistance in crops will be reviewed. These will include mutant selection in cultured cells, transfer of chloroplasts via protoplast fusion, and selection of recombinant chloroplast genomes. Examples will be limited to resistance to triazine herbicides. Chloroplast transformation involving introduction of novel DNA into chloroplasts, will not be covered. Preliminary data indicate that chloroplast transformation may be feasible (4) but have not been confirmed. The genetics, physiology

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and agricultural implications of herbicide resistance in crop and weed species have been reviewed extensively (1,2,5,6).

Selection of Chloroplast-encoded Mutants

Triazine-resistant weeds have appeared in fields where triazine herbicides have been continuously used. At least 38 resistant weed species have been identified (2). The weed biotypes are resistant to a number of triazine herbicides, including atrazine and terbutryn, and have a characteristic, altered spectrum of sensitivity to other herbicides (1). Resistance in *Amaranthus hybridus* (7) and *Solanum nigrum* (8) has been attributed to a mutation in the chloroplast *psbA* gene. The frequency of appearance of resistant biotypes has been calculated to be very low (1). It was therefore proposed that a nuclear-encoded chloroplast mutator gene was involved (1).

Because of the expected low frequency of appearance of resistant biotypes, there has been some skepticism regarding the possibility of inducing chloroplast-encoded herbicide resistance mutants in higher plants. This skepticism is based on the known high genome copy number in the chloroplasts and the high number of chloroplasts per cell (9). However, identification of chloroplast-encoded streptomycin- and lincomycin-resistant mutants indicated that it is possible to overcome the problems of chloroplast population, and *Nicotiana* plants have been produced that carried a uniform population of mutant chloroplast genomes (10).

Triazine-resistant mutants have now been isolated in protoplast cultures of *Nicotiana plumbaginifolia* (11). The key to recovering the triazine resistance mutants was the use of photomixotrophic cell cultures. Photomixotrophic cultures were obtained by reducing the sucrose concentration from 3% to 0.3%. On this medium photobleaching by terbutryn, a triazine herbicide, could be observed in cell culture. Resistant cell lines were identified on a selective medium (10^{-4} M terbutryn) by their green pigmentation in the population of bleached, sensitive calli. Maternal inheritance of the resistance phenotype, the spectrum of cross-resistance to other herbicides, and the extreme resistance to triazine herbicides *in vitro* in electron transport assays suggested (11) that the mutants obtained in cell culture were similar to the resistant weed biotypes.

Triazine-resistant mutants have also been isolated in cell cultures of *Nicotiana tabacum* (12). However, the mutants have not been characterized to date.

In higher plants altered photosynthetic electron transport in the triazine-resistant mutants (2,13) has been correlated with slower growth and lower yield (14). Experiments with *Chlamydomonas*, a unicellular alga, suggest that triazine resistance is not necessarily associated with an alteration of the photosystem II electron transport kinetics (15). Selection of mutants resistant to other classes of photosystem II inhibiting herbicides, (e.g. diuron, bromacil) should also be feasible based on successful isolation of such mutants in *Chlamydomonas* (16).

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Chloroplast Transfer

The chloroplasts of triazine-resistant weed species are a potential source of herbicide resistance for transfer into crop plants. Chloroplast transfer, that is the combination of desirable chloroplasts and nuclei, can be achieved by crossing or by protoplast fusion. Chloroplast transfer by crossing is carried out using the line with the desirable chloroplasts as the maternal parent and the line with the desirable nuclear background as the pollen parent. Repeated crossing results in substituting the chromosomes (nucleus) of the chloroplast source with those of the recurrent pollen parent as chloroplasts in crops are only inherited by the maternal parent. This strategy has been used to introduce the triazine-resistant chloroplasts from bird's rape (a weed, *Brassica campestris*) into cultivated *B. campestris* and *B. napus* (3).

Protoplast fusion provides an alternative method for chloroplast transfer (17,18). Transfer is based on the independent segregation of chloroplasts and nuclei in heterokaryons obtained by protoplast fusion. Efficiency of recovery for specific combinations of chloroplasts and nuclei has been facilitated by using selectable chloroplast markers such as streptomycin or lincomycin resistance, or by using maternal pigment-deficient mutants to visually differentiate between clones carrying donor and recipient chloroplasts. Elimination of the nucleus of the chloroplast donor is facilitated by irradiation of donor protoplasts prior to fusion. Methods of chloroplast transfer by protoplast fusion have been reviewed (17,18). In this review only transfer of triazine-resistant chloroplasts will be covered. Work in *Nicotiana* is more recent than in rapeseed (19) and potato (20,21), but serves to illustrate the methodology of chloroplast transfer by protoplast fusion.

Chloroplast transfer in Nicotiana. Terbutryn resistance has been reported in cell cultures of *N. plumbaginifolia* (11), a weed species known to be cross-fertile with cultivated tobacco (*N. tabacum*). Biochemical and biophysical characteristics of the TBR2 line suggested that resistance was due to a mutation in the chloroplast *psbA* gene. While TBR2 chloroplasts could be introduced into *N. tabacum* by repeated backcrosses between the TBR2 (*N. plumbaginifolia*) mutant and *N. tabacum*, this would result in an alloplasmic substitution line, carrying the *N. tabacum* nucleus and *N. plumbaginifolia* cytoplasm, that would be male sterile (22). Triazine resistance (a chloroplast trait) and male sterility (a mitochondrial trait) cannot be separated by crossing. It has been shown, however, that after protoplast fusion, independent segregation of organelles in cytoplasmic hybrids (cybrids) can result in new combinations of chloroplast and mitochondrial traits (23,24). In this section we describe previously unpublished work on the transfer of TBR2 chloroplasts into a *N. tabacum* nuclear background by protoplast fusion and discuss the characteristics of the resulting cybrid plants.

Chloroplast transfer was carried out using the albino *N. tabacum* recipient, SR1-A15 (25), in which pigment deficiency is

controlled by a plastid gene mutation. The chloroplast donor TBR2 was X-irradiated using a dose that was sufficiently high to prevent cell division but allowed recovery of TBR2 chloroplasts following fusion with SR1-A15 cells. *N. tabacum* clones carrying recovered TBR2 plastids were identified as green colonies among the pigment-deficient colonies derived from unfused SR1-A15 cells (Fig. 1).

Diploid *N. tabacum* cybrids carrying the triazine-resistant chloroplasts of the TBR2 line were obtained at a high efficiency. Plants studied in twelve out of eighteen independent clones were the right combination of chloroplasts and diploid *N. tabacum* nuclei. Several factors contributed to the efficiency of cybrid selection: irradiation of the chloroplast donor facilitated elimination of the donor nucleus at the heterokaryon stage, the use of an albino mutant as a chloroplast recipient facilitated identification of clones with TBR2 plastids, and preselection by morphology helped eliminate somatic hybrids and polyploid forms.

Independent segregation of chloroplasts and cytoplasmic male sterility (a mitochondrial trait) has been reported in *N. tabacum* nuclear background after fusing lines carrying normal (fertile) *N. tabacum* cytoplasm with lines carrying *N. debneyi* (23) or *N. undulata* (24) cytoplasms. Cybrid clones both with normal fertile flower morphology and with male-sterile flowers were therefore expected in our experiment. Instead, flower morphology in each of the twelve clones was abnormal. It was similar to that of the alloplasmic subsitution line carrying the wild-type *N. plumbaginifolia* cytoplasm (22), a morphology that results in functional male sterility even if the pollen produced on the anthers is fertile. The stigma in each clone was protruding above the lips of the corolla and the filaments were shorter than normal. Presence of *N.plumbaginifolia* (TBR2) chloroplasts as the source of abnormality could be excluded since *N. tabacum* cybrids derived from the same species combination, but carrying *N. tabacum* chloroplasts have the same flower morphology. Finding cybrids with a similar flower morphology in all twelve clones is explained by the preferential maintenance of certain mitochondrial recombinant classes (18). This observation places a limit on the use of *N. plubaginifolia* chloroplast mutants in *N. tabacum* unless contribution by the *N. plumbaginifolia* mitochondrial genome can be prevented.

The original TBR2 plants have reduced vigor. Reduced vigor could be due to genetic chages in the nucleus induced by cell culture (26), or could be a consequence of the plastid gene mutation conferring triazine resistance (2,14). Chloroplast transfer did not result in significantly improved viability of the triazine-resistant plants. The cybrids exhibited a tendency for chlorosis and grew slower than wild-type plants, characteristics similar to those of spontaneous atrazine-resistant weed mutants (14). These symptoms are probably the consequences of impaired photosynthetic electron transport (13,14) and are linked to the chloroplast mutation. Incompatibility of *N. plumbaginifolia* chloroplasts with the *N. tabacum* nucleus cannot be the source of these problems since the symptoms are absent in the alloplasmic substitution line.

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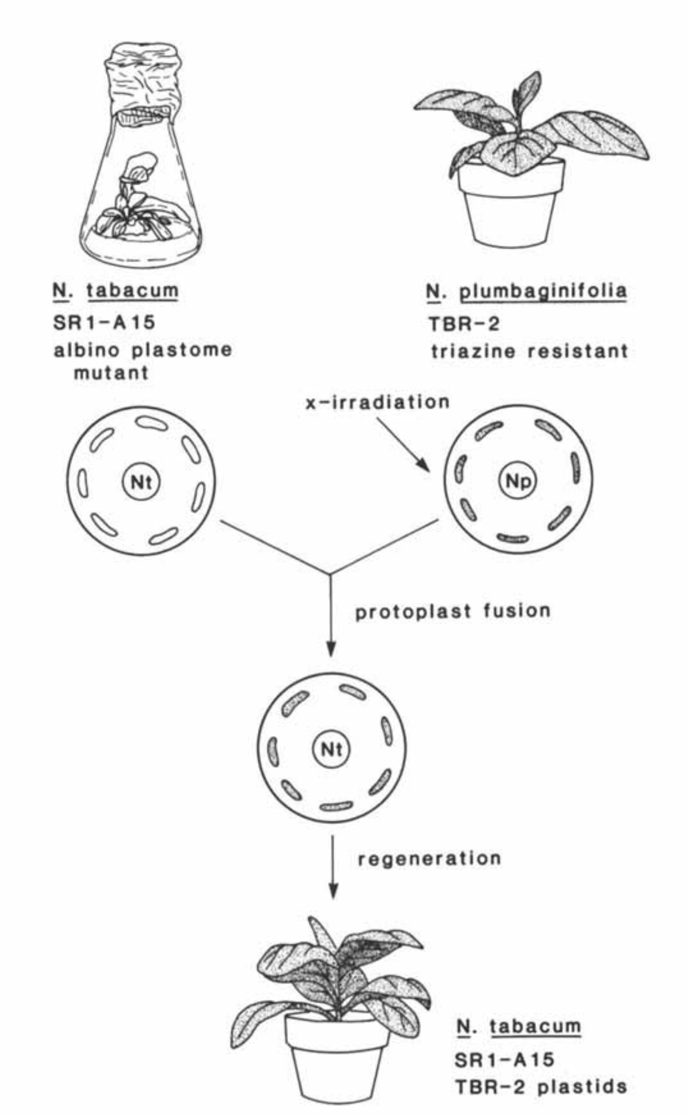

Figure 1. Strategy for the transfer of atrazine-resistant chloroplasts from N. plumbaginifolia into N. tabacum.

N. tabacum plants with TBR2 plastids survived treatment with higher doses than 10 kg/ha whereas plants with sensitive *N. tabacum* plastids were killed by 1 kg/ha atrazine (Fig. 2). Transfer of the TBR2 chloroplasts into the nuclear background of a different species did not affect the expression of the resistance. The level of atrazine resistance seems to be sufficiently high to protect the crop under field conditions since atrazine is usually applied at a rate of 2-4.5 kg/ha.

Combination of atrazine resistance and cytoplasmic male sterility in rapeseed. Transfer of atrazine resistant chloroplasts from bird's rape (*B. capestris*) into oilseed rape has been accomplished by back-crossing (3, this volume). The purpose achieved by somatic cell fusion was to combine the atrazine-resistant chloroplasts carried by a *B. napus* line with cytoplasmic male sterility (CMS), a mitochondrial trait (19). The CMS trait was carried by a *B. napus* line with the cytoplasm of radish (*Raphanus sativus*). Since both traits, atrazine resistance and CMS, are maternally inherited in sexual crosses, the only way to combine the traits was by protoplast fusion. The desired atrazine-resistant cybrids were identified in a randomly regenerated sample of plants by the presence of the CMS flower morphology and their darker green color at low temperature. Somatic hybrids rather than cybrids were obtained in each clone since no irradiation had been used to facilitate the elimination of one of the parental nuclei. Chromosome numbers were subsequently reduced to the diploid level by crossing.

Chloroplast transfer into potato. Transfer of atrazine-resistant *Solanum nigrum* chloroplasts into *S. tuberosum* has been attempted by protoplast fusion (20,21). In the absence of irradiating the chloroplast donor *S. nigrum*, only somatic hybrids were obtained (21). There has been no further report on these plants. In any case, the somatic hybrid lines could not be commercially utilized even if they were fertile. Crosses using a potato line as a recurrent pollen parent do not result in an alloplasmic substitution form of the pollen parent, because potato cultivars cannot be maintained by seed due to their heterozygosity.

Chloroplast Recombination

The ancient art of plant breeding is based on combining favorable genes from different parental lines. One such gene could be that for chloroplast-encoded herbicide resistance. In crop plants chloroplast traits have been excluded from this strategy as they are inherited exclusively by the maternal parent (27,28). Since no mixed organelle populations are formed, there is no opportunity for genetic recombination between the parental organelle genomes. No chloroplast gene recombination has been detected in higher plant species in which chloroplasts are inherited biparentally (29,30). Lack of detecting chloroplast recombinants in this case has been attributed to their low frequency of occurrence (30). Recombination between chloroplast markers has been extensively demonstrated, however, in the unicellular alga *Chlamydomonas reinhardii* (28).

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Figure 2. Atrazine resistance of N. tabacum cybrids Nt(TBR2)4 (top row). Wild-type N. tabacum is also shown (bottom row). Application rate of atrazine (Aatrex, CIBA-GEIGY) was (left to right) 1, 10, 100 and 1000 kg/hectare.

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Genetic data have been corroborated by physical evidence for chloroplast recombination (31,32). These data suggested that, given an opportunity for chloroplast mixing, recovery of chloroplast recombinants is feasible in flowering plants.

Plant cell culture techniques have provided the tools to enable successful recovery of chloroplast recombinants. These include utilization of selectable chloroplast markers such as resistance to the antibiotics streptomycin and lincomycin (10) and efficient methods for somatic cell fusion that enable the production of large numbers of cells with mixed chloroplast populations (33).

Initial analysis of the chloroplast DNA from somatic hybrids and somatic cybrids with mixed chloroplast populations failed to reveal recombinant chloroplast genomes. Organelle segregation resulted in plants with either parental chloroplast type (23,24,34). To date, only a single chloroplast recombinant has been reported in flowering plants, in line pt14, a somatic hybrid of *N. tabacum* and *N. plumbaginifolia* (25). Isolation of this recombinant was facilitated by the use of a *Nicotiana tabacum* line, SR1-A15, with chloroplast genomes carrying multiple markers. The SR1-A15 line is a derivative of the SR1 maternal streptomycin-resistant mutant and carries an additional maternally-inherited mutation that prevents the expression of streptomycin resistance, i.e., greening on streptomycin. Selection of putative chloroplast recombinants was based on the expression of streptomycin resistance as the result of the correction of the pigment mutation via recombination with the chloroplast genome of a streptomycin-sensitive (and licomycin-resistant) *N. plumbaginifolia* line, LR400. Physical mapping indicated that the chloroplast genome contains at least six recombination sites (25). Endonuclease restriction digests of cloned parental and pt14 chloroplast DNA clones with a more extensive set of enzymes has led to the conclusion that the recombinant genome contains many more recombination sites than was initially assumed (35). A detailed study of the 17.1 kb *Sal* I fragment revealed recombination sites every few kilobases in the recombinant chloroplast chromosome (unpublished). These findings suggest that higher plant chloroplasts have an active recombination system. The rate limiting step in the formation of recombinant chloroplasts may therefore be the formation of heteroplasmic chloroplasts, most likely by plastid fusion.

The most important use of chloroplast recombination will be the combination of chloroplast genes from different organisms. The chloroplast genomes of closely related species, such as *N. tabacum* and *N. plumbaginifolia*, are very similar, and homologous recombination should be feasible in any region of the genome. Even among unrelated plant species, however, at least 30% of the chloroplast DNA sequences are shared. These conserved sequences are interspersed with divergent ones, and probably correlate with coding and non-coding regions (36). Chloroplast recombination therefore may be feasible between heterologous chloroplast genomes.

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Concluding Remarks

Cell culture techniques for modifying the chloroplast genome in flowering plants have only been recently developed. Plants with chloroplast-encoded herbicide resistance obtained by these methods will most probably be a modest use of these technologies compared to the impact of improving the photosynthetic efficiency or stress tolerance of crop plants. The potential value of herbicide resistant forms, however, is significant enough to justify further refining the methods for chloroplast mutant selection, chloroplast transfer, genetic recombination, and transformation of chloroplasts. Research with photosynthetic bacteria and with the unicellular alga *Chlamydomonas* will continue to provide analogies and examples for future research with higher plants (37).

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Chapter 10

Developing Insecticide Resistance in Insect and Mite Predators and Opportunities for Gene Transfer

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Genetic selection of predatory insects and mites for pesticide resistance has been successful and resistant strains of the western predatory mite, *Metaseiulus occidentalis*, are being commercially implemented in California almond orchards. Multi-resistant strains of *M. occidentalis* were developed that are resistant to carbaryl, sulfur, permethrin and organophosphorus insecticides. The common green lacewing, *Chrysoperla carnea*, also was selected in the laboratory and this predatory insect developed a high level of carbaryl resistance. Whether parasitoids can be selected for a high level of resistance remains unknown. However, the development of resistant parasitoids and predators could lead to their enhanced use in agricultural crop systems where key insect pests must be controlled chemically. Because pesticide resistances are often determined by single major genes, gene transfer techniques should be feasible if the resistance genes can be identified, cloned, transferred to recipient species with a suitable vector, stably and appropriately expressed in the recipients, and transmitted to their progeny.

Biological Control and Agricultural IPM Systems

Biological control of arthropod pests by parasitoids and predators is a cost effective and, potentially, permanent form of

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pest control (1-2). Unfortunately, many arthropod natural enemies are more susceptible to pesticides that are the target pest arthropods (3-5), which may result in secondary pest outbreaks. One method for integrating biological control in agricultural integrated pest management (IPM) systems involves the use of selective pesticides. Such selective pesticides should be used in a manner so as to be less toxic to the nontarget biological control agents. Tactics to achieve this goal include the choice of selective pesticides, selective placement, timing of applications, reductions in rates applied, and choice of application methods (4). A special form of pesticide selectivity involves the use of pesticide-resistant natural enemies. Unfortunately, pesticide resistances develop rarely in the field in arthropod natural enemies (6). Reasons for this differential response are no doubt due to biological, ecological, and, perhaps, physiological factors (4-5). Thus, laboratory selection or genetic engineering for resistances in parasitoids and predators is a logical direction to take to increase the use of arthropod biological control agents in agricultural IPM systems (7-8).

Genetic Improvement

Genetic manipulation of arthropod natural enemies is an old concept (9-10), although field implementation of genetically-improved natural enemies was not achieved until recently (8,11). Various biological and behavioral attributes have been discussed as amenable to genetic selection, including sex ratio, temperature tolerance, developmental rate, fecundity, diapause, and host preference, as well as pesticide resistances (12). Initiating a genetic improvement program requires that the factor(s) limiting the efficacy of the natural enemy be identified before "improvement" can take place (8,12). Rarely do we understand the detailed biology and ecology of arthropod natural enemies sufficiently well that we can identify the critical component(s) limiting the natural enemy's effectiveness. However, pesticide susceptibility is often a clearly limiting factor for natural enemies in agricultural IPM systems and enhancing their survival after pesticide applications can often lead to more effective control of both target and nontarget pests in agricultural crop systems. Since pesticide resistances (13) are often determined by a single major gene rather than several, genetic improvement projects are somewhat easier to implement since maintenance of the resistance and management of the resistant strain after release into the field is simplified (8). This is particularly important when releases occur into an area in which susceptible native strains exist; if a resistant strain with a polygenically-determined resistance interbreeds with a susceptible (native) strain, the resistance is rapidly lost unless intense pesticide selection pressure is maintained.

Resistances in Parasitoids

Several parasitoids have been selected for pesticide resistance. The parasite Aphytis melinus(Fig 1) became 3.4 times more resistant to malathion after 8 generations of laboratory selection (14). Unfortunately, this increase is insufficient to be of practical value since the red scale pest is 707 times as tolerant to malathion as the original A. melinus colony. Havron (15) attempted to select the parasite Aphytis holoxanthus for resistance to malathion, but was unable to obtain a stable or high level of resistance despite 30 selection cycles. Strawn (16) did find variability in responses to four organophosphorus insecticides among colonies of A. melinus and Comperiella bifasciata collected from California citrus orchards but did not select these colonies. Schoonees and Giliomee (17) found that one geographic strain of Aphytis africanus differed from another in its tolerance of methidathion by a factor of 5.7. Similarly, Comperiella bifasciata was 65.6 times as tolerant of methidathion as a laboratory culture. This variability was correlated with differential use of pesticides in the collection areas. Schoonees and Giliomees (17) concluded, however, that these levels were insufficient to allow adult parasitoids to survive in the field as the field rates are 17-25 and 12-18 times higher than the LC_{50} values for A. africanus and C. bifasciatus, respectively.

Parasitoids may be unable to develop high levels of pesticide resistance since they have lower levels of mixed function oxidases than phytophagous arthropods (5), although they may be well-adapted to hydrolytic detoxification pathways through esterases. Adams and Cross (18), Pielou and Glasser (19), and Robertson (20) also failed to obtain levels of pesticide resistance that would allow the parasitoid to survive under field conditions, and the question thus remains as to whether parasitoids have the biochemical and genetic capability to develop high levels of resistance to pesticides.

Because resistance to insecticides could be achieved through several different mechanisms other than mixed function oxidases or esterases, additional options should be investigated. These include the development of behavioral resistance, target site insensitivity, change in rate of penetration, and rate of storage. There is no reason to conclude that parasitoids could not develop resistances through one or more of these mechanisms. Various ecological and behavioral factors also limit the rate of resistance development in arthropod natural enemies and these difficulties could be overcome in the laboratory during selection programs. To date, few selections have been conducted with parasitoid populations that were derived from large, genetically diverse sources. Most colonies subjected to selection had been held in the laboratory for long periods or appear to have been initiated with only a few individuals. Such limitations on genetic variability would limit the response to selection. Current work conducted at the University of California at Berkeley by J. A. Rosenheim (personal communication) with Aphytis melinus suggests that such large samples provide important genetic variability in responses to 6 pesticides. Whether

Figure 1. The parasitoid Aphytis melinus parasitizes a California red scale insect. A. melinus is being selected for pesticide resistances. (Photo by Jack Kelly Clark, U.C. Cooperative Extension.)

such variability will provide the basis for a selection for a large scale resistance remains to be determined. If artificial selection for resistance is ineffective, perhaps molecular gene transfer techniques can be applied to parasitoids. Thus, several areas of research remain unexploited (7).

Resistances in Arthropod Predators

Several predatory insects and mites have developed pesticide resistances through laboratory selection and the resistances are sufficiently high that they can survive field application rates. The most extensive research has been conducted with predatory mites in the family Phytoseiidae (Figure 2) (for a review, see (8)). These predatory mites are excellent predators of spider mites (Tetranychidae) in diverse agricultural crop systems around the world (21-23). After World War II, spider mites became serious agricultural pests, in large part due to the use of synthetic pesticides which killed their predators (21). Gradually, it became apparent that certain populations of phytoseiids had acquired resistances to organophosphorus (OP) insecticides in the field (6,4), and these insecticides were used preferentially in IPM systems to control insects so that disruption of biological control of the spider mites did not occur. The OP resistance in these predatory mites does restrict the flexibility of IPM programs and the idea of selecting for additional resistances in the laboratory developed. Schulten and van de Klashorst (25) selected the phytoseiid predator *Phytoseiulus persimilis* for parathion resistance. By the time the selection was completed,

Figure 2. The predatory mite *Metaseiulus occidentalis* feeds on a two-spotted spider mite egg. *M.occidentalis* has been selected for carbaryl and permethrin resistances in the laboratory and OP- and sulfur-resistant strains have been crossed to produce multi-resistant strains. (Photo by Jack Kelly Clark, U.C. Cooperative Extension.)

however, a naturally-occurring OP-resistant strain was discovered and became used in commercial mass production and releases. Additional laboratory selection projects have since been conducted with *Metaseiulus occidentalis*, *P. persimilis*, *Amblyseius fallacis*, and *Typhlodromus pyri* (8).

Amblyseius fallacis. *A. fallacis* is an important predator of spider mites in deciduous orchards in the eastern U.S.A. and its field-developed resistance to OP insecticides has been augmented by laboratory selection for carbaryl and permethrin resistances (26-27). The carbaryl resistance appeared unstable and is not currently being used. Permethrin resistance is polygenically determined and the selected strains have undergone field trials (28).

Metaseiulus occidentalis. The western predatory mite, *M. occidentalis*, has been selected for resistances to carbaryl, methomyl, dimethoate, carbaryl, permethrin, and fenvalerate in the laboratory (8). Carbaryl, OP, and sulfur resistances are determined by major semidominant genes, while permethrin and fenvalerate are multigenically-determined. Strains have been developed that are multiply-resistant (29), and these have undergone field testing (11, 30-33). The multiresistant strains have been tested in both individual tree plots and in large commercial orchards. In both situations, the predators established, overwintered, survived pesticide applications, and controlled spider mites. Two mass rearing methods were developed (34), along with an implementation system for almonds in California. Several companies are producing the predators commercially and providing management advice for a fee to almond and apple growers in California and British Columbia.

Other Phytoseiid species. *Typhlodromus pyri* and *Phytoseiulus persimilis* are undergoing selection for resistance to pyrethroid insecticides (N. Markwick, personal communication), and field trials of the selected strains were scheduled to occur during the 1985 field season in New Zealand (H. Wearing, personal communication).

Fitness of Selected Resistant *M. occidentalis* Strains

Genetic improvement projects could result in strains with decreased fitness through the inadvertent selection of deleterious genes, or because the resistance gene(s) themselves confer lowered fitness. Such deleterious pleiotropic effects are considered to be common, or the resistance allele would be the "wild type". The most detailed examination of relative fitness of resistant and susceptible strains has been conducted with *M. occidentalis*.

The carbaryl-OP resistant strain of *M. occidentalis* was evaluated in the laboratory, greenhouse and in the field (35).

Development time, fecundity, sex ratio, mating compatibilities and competition, diapause, persistence of the carbaryl resistance, and the capability to control spider mite populations under carbaryl sprayed and unsprayed conditions were evaluated. The resistant strain did not differ significantly from the susceptible strains tested in the absence of carbaryl treatment.

The carbaryl-OP-permethrin, carbaryl-OP-sulfur, and non-diapause-carbaryl-OP resistant strains also were evaluated in the laboratory (29). These multiresistant strains were not different in their fecundity compared to a wild strain, although longevity of adult females varied, with the wild females having a reduced longevity. Developmental rates did not differ substantially, and sex ratio was variable, but not worse in the multi-resistant strains. Field evaluations of the carbaryl-OP-sulfur resistant strain (30,32-33), the OP-permethrin (31), and carbaryl-OP-nondiapause strain (36) support the conclusion that the genetically-manipulated strains are sufficiently fit that they can be considered to be genetically improved, particularly with regard to their ability to survive these pesticides. In the field, the carbaryl-OP and carbaryl-OP-sulfur resistant strains survive these pesticides, establish in the orchard or vineyard, overwinter, and control spider mites. Thus, releases of these resistant strains are inoculative, and long-term (at least five years) persistence can occur (30,32-33). The permethrin-OP resistant strain can only survive low rates of permethrin, but it is able to persist over several seasons in apple and pear orchards (31).

Mechanisms of Resistance in Phytoseiids

An OP resistant strain of *Amblyseius fallacis* degraded more azinphosmethyl than did a susceptible strain, both *in vivo* and *in vitro* (37). The major metabolite was identified as desmethyl azinphosmethyl, and the resistance is attributed at least in part to the higher rate of desmethylation, but is not associated with a modified cholinesterase. The resistant strain had a higher nonspecific esterase activity than the susceptible strain; no difference in penetration of carbon-14 labelled azinphosmethyl was found. Carbaryl and propoxur resistance in *Metaseiulus occidentalis* is related to high levels of oxidative detoxification, which is present even in the egg stage (38). The mode of inheritance for the resistances in both species is due to a partially dominant single gene. Scott et al. (39) evaluated several *Amblyseius fallacis* strains resistant to permethrin and showed that a mixed-field strain had a *kdr* type of resistance. Permethrin resistance is multi-genetically determined in *A. fallacis*. Esterase activity varied in several permethrin-resistant strains, as well. Parathion and propoxur resistance in *Typhlodromus pyri*, a strain in which resistance is dependent on a semi-dominant gene, is due to insensitivity of acetylcholinesterase (40). No information is available on the mechanism of resistance to sulfur in *M. occidentalis* (8).

Resistance in *Chrysoperla carnea*

Chrysoperla carnea (Fig 3), the common green lacewing, is an insect predator of aphids, mites, and small insects. Grafton-Cardwell and Hoy (45) documented that intraspecific variability in responses to pesticides occurred among different populations collected from different geographic regions in California. Such variability was used as the basis of a selection program and a high level of resistance to carbaryl was achieved by selecting first instar larvae (46). After only four rounds of selection, larvae survived rates of carbaryl used in the field. Mode of inheritance analyses demonstrated that carbaryl resistance does not fit a model of a single dominant gene. Tests with the oxidase inhibitor piperonyl butoxide and the esterase inhibitor phenyl saligenin cyclic phosphonate indicated that both oxidase and esterase enzynes contribute to the resistance. The resistant strain exhibited lower larval and pupal survival and produced fewer females than the colony it was derived from under carbaryl-free conditions. However, its fecundity was significantly higher and adult longevity was higher. These attributes could compensate for the reduced survival of the immature stages in untreated conditions. This is the first insect predator that has undergone successful selection for a high level of pesticide resistance in the laboratory.

Implementation of Pesticide-Resistant Strains

Biological control typically employs three tactics: 1) classical importation and establishment of exotic natural enemies to control exotic pests, 2) conservation of natural enemies, and 3) augmentation through mass rearing and releases (1). Genetic improvement of biological control agents can be employed in tactics 1 and 3 (12). Developing pesticide- resistant strains of parasitoids and predators and their long term establishment may result in their conservation (tactic 2) if the natural enemy becomes permanently established in a new environment after release.

Classical establishment of resistant natural enemies requires mass rearing and releases to achieve establishment. A quality control program is crucial to ensure the released strains are fit and effective. Additional releases should then be required only when new geographic sites require releases or if new pesticides are introduced to which the natural enemy(ies) is not resistant. If releases are being made into an area in which no susceptible natives exist, the genetic basis of the resistance is not important, as long as it is stable. However, if resistant strains are being released into areas in which susceptible natives exist which can interbreed with them, special precautions must be taken to ensure the resistant strain establishes and persists. This may require substantial knowledge of the dispersal and population dynamics of the species involved. Resistances determined by single, major dominant genes are clearly the easiest to manage in such

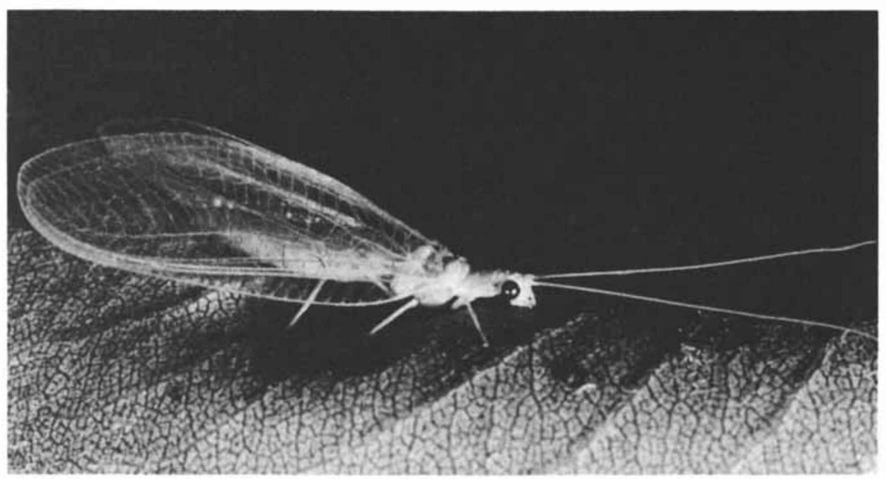

Figure 3. The common green lacewing Chrysoperla carnea preys, as immatures, on small arthropods. C.carnea was selected for carbaryl resistance in the laboratory. (Photo by Jack Kelly Clark, U.C. Cooperative Extension.)

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circumstances. Strains with polygenically-determined resistances that are being released into a new geographic area do not require special care, but when such strains are being released into an area with susceptible natives with which they can interbreed, special measures may be required to retain the resistances, particularly if the predator or parasitoid has a high dispersal rate (8). At the present, we know too little about the population dynamics of most biological control agents to be able to predict the outcome of such releases. Computer models may provide much needed information about optimal release rates and timing, appropriate timing and numbers of pesticide applications, diffusion of resistance genes due to interbreeding and dispersal, or at least provide clues as to the critical data needed to carry out a practical implementation program (41-42). Thus implementation of genetically-improved biological control agents will require substantial knowledge and effort; acquisition of this necessary biological and ecological information may require investments in research equivalent to, or greater than, that invested in the genetic manipulation (43).

Economic Analysis of a Genetic Improvement Program

Headley and Hoy (43) developed a cost-benefit analysis of the genetic improvement project with *M. occidentalis*. The carbaryl-OP-sulfur resistant strain is being implemented in California almond orchards as part of an integrated mite management program. The implementation components took at least 3 years to develop-longer than the genetic selection portion of the project. Research conducted to enable implementation involved developing two mass rearing methods, and two sampling methods so that predators and spider mites can be monitored to determine that effective control is being provided by the predators. Furthermore, research was required so that if the predators are providing insufficient control, the predators can be assisted through the use of low rates of selective acaricides which partially suppress spider mites but has little impact on the predators. In addition, the project requires that the growers use azinphosmethyl, diazinon, phosmet or carbaryl for the control of the key insect pest, the navel orangeworm, as these are selective for the resistant strains of *M. occidentalis*. Despite the relative complexity of the program, as compared to a conventional chemical control program, almond growers are adopting it because of the large cost savings they can achieve through the reduced numbers of applications of acaricides and the lower rates applied. The cost of the program includes $20 per acre for predator releases (if needed), monitoring costs of about $10 per acre, and applications (as needed) of low rates of acaricides to all or part of the orchards. Despite these costs, savings of $50-60/acre, or more, are possible. Risks are low, since the grower can simply apply a "normal" rate of acaricide if the predators for some reason do not perform well. Native pesticide-resistant strains of *M. occidentalis* are estimated to be very valuable, as well. The native, OP-resistant strain of *M. occidentalis* used in apple orchards in Washington (24) are estimated to reduce the amount spent on pesticides and application for control of spider

mites by $5,000,000/year (44). Thus, the value of native or laboratory-developed pesticide-resistant predatory mites is evident, whereas the value of pesticide-resistant insect predators or parasitoids remains uncalculated because none have yet been implemented in IPM programs.

Potential for Gene Transfer

The genetic improvement projects described above utilized traditional genetic selections for pesticide resistance. Such selection projects are limited by the presence of sufficient variability in the initial colony undergoing the selection. If relevant resistance genes are not sampled, or if they don't exist in the species of interest, the genetic improvement project is doomed.

A solution to the sampling problem, and to the apparent lack of resistance genes of interest in the target beneficial species, might be to use molecular cloning techniques to insert pesticide resistance genes from one species into different beneficial insect or mite species (7).

Genetic engineering techniques have been developed for one insect -- *Drosophila melanogaster* (7). Thus, it may be possible to isolate resistance genes from one species from a library with the complete DNA of the species. Once a gene is isolated, it may be possible to transfer it into the genome of a new species. At the moment, *Drosophila* species are the major insects into which a cloned gene can be introduced so that it is properly expressed (47-49). The introduction requires a transposable P element to serve as a carrier, or vector, of the foreign DNA. Thus, a cloned P element is injected into an early *Drosophila* embryo which comes from an M strain so that the P element will transpose at a high frequency into the resident chromosomal DNA. When cloned genes are placed between the ends of the P element, they are inserted into the chromosomal DNA along with the P element (47). In most cases, the regulation of genes introduced by P-element transformation is normal and unaffected by the position of insertion into the chromosome (48). Recently, the P element was used to mediate transformation into other species of *Drosophila* (40). Whether the P element can be used to transpose genes into species outside the genus *Drosophila* remains to be determined (51). However, it is likely that ongoing research to improve the host range of the P element will provide new and improved opportunities to use the P element as a vector to introduce pesticide resistance genes into beneficial insect species (G. Rubin, personal communication). Or, other vectors may be developed from other insect species. Much research remains to show that gene transfer techniques can provide useful strains of biological control agents. A potential benefit of such research is the possibility that transfer of a single, well characterized, resistance gene will result in strains which do not suffer from the deleterious effects of inadvertent selection for reduced fitness. Furthermore, gene transfer techniques may make genetic manipulation of biological control agents more efficient and predictable in terms of the level of resistance achieved, which would make such projects more cost effective.

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Chapter 11

Importance of Conformational Variability in Protein Engineering of Subtilisin

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Enzymes are highly specific catalysts. The nature of this specificity is believed to result from structural and electrostatic complementarity between the enzyme and its substrate. The serine protease, subtilisin, is being extensively studied as a model system to explore the effects of single amino acid substitutions on its structure and function (1). The gene for Bacillus amyloliquefaciens subtilisin has been expressed and secreted in B. subtilis (2). A site-directed mutagenesis scheme, cassette mutagenesis (3), has been used to produce a series of subtilisin variants that are more resistant to oxidants (4), and have altered stability (5), specificity, and specific activity.

Here we report the analysis of the functional and structural effects of single amino acid substitutions at position 166 where a glycine is found in all the sequences of naturally occurring subtilisins from a number of Bacillus species. All substitutions have measurable activity. Substitutions of asparagine or lysine for glycine give variant enzymes with higher specific activity or altered specificity, respectively. The three-dimensional structure of B. amyloliquefaciens subtilisin, originally determined at 2.5 A resolution (6,7), has now been refined at 1.8 A resolution (8). Two candidate variants, asparagine and lysine, were chosen for study using X-ray crystallography. The three-dimensional structures of these position 166 variants show a pattern of localized structural perturbations rather than global conformational change. The alterations of specificity in these variants appears to be linked to the degree of side chain rigidity, which affects the distribution of electrostatic charge at the substrate binding site.

Molecular Modeling at Position 166

Previous studies have demonstrated that subtilisin has a chymotrypsin-like specificity (reviewed in 9), preferring to hydrolyze the peptide bond following a large hydrophobic residue. A model of substrate binding has been deduced from the three-dimensional models of peptide and protein inhibitor complexes with subtilisin (10). The model we have derived is shown in Figure 1. The enzyme can be divided in a series of subsites

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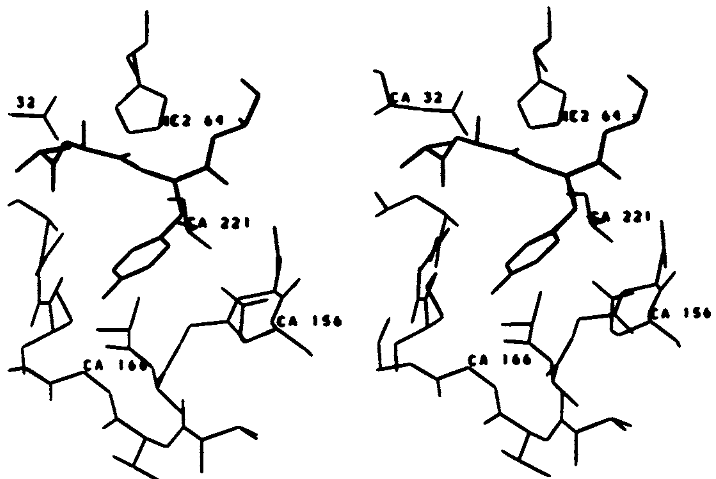

Figure 1. Stereoscopic view of the subtilisin active site. The catalytic residues asp 32, his 64 and ser 221 along with glu 156 and gly 166 are labeled. A substrate model (dark lines) having tyrosine at the P1 position has been positioned to indicate the S1 position (see text) of the enzyme.

following the nomenclature of Berger and Schecter (11). (Side chains of the peptide substrate are labeled with respect to the scissile peptide bond where P1, P2... extend toward the N-terminus and P1', P2'... extend toward the C-terminus, the corresponding binding sites on the enzyme are labeled S1, S2... and S1', S2'...). The S1 binding site is an open trough rather than a cavity or "pocket". Modeling of residues other than a glycine at position 166 suggested that a substitution at this site would likely close off the S1 binding site to form more of a "pocket" as seen in the S1 site in chymotrypsin for example (12). The modeling further suggested that 166 was a "forgiving site", that is, a number of substitutions could be accommodated with stereochemically reasonable conformations.

Results of Mutagenesis

Substitutions at position 166 were introduced using cassette mutagenesis (3). First, unique and silent restriction sites, flanking codon 166, were introduced by oligonucleotide directed mutagenesis; cassettes of synthetic duplex DNA oligonucleotides could then be ligated to replace the natural coding segment excised by the appropriate restriction enzymes. Plasmids containing the altered *B. amyloliquefaciens* subtilisin gene were expressed in a protease deficient strain of *B. subtilis* to insure that the only functional subtilisin originated from the plasmid born gene. The proteins were purified from *B. subtilis* culture supernatants by published methods (4) and the proteolytic activity on casein for each of the nineteen possible variants at position 166 was measured. The results showing the rate of casein hydrolysis at a fixed enzyme and substrate concentration for 20 min are presented in Table I. Under these conditions one variant, Asn 166, showed greater activity than the native enzyme. All variants had measurable activity at the reaction conditions described in Table I, indicating that glycine is not strictly required for activity.

In order to probe the underlying kinetic alterations that give rise to the variation in overall proteolytic activity, the kinetic parameters (K_m, k_{cat}, k_{cat}/K_m) of a series of synthetic substrates were determined. K_m for amide hydrolysis can be interpreted as the dissociation constant for the Michaelis complex (E·S), and k_{cat} as the acylation rate constant. The substrates are of the form: succinyl-Ala-Ala-Pro-X-p-nitroanilide, where X is the P1 amino acid. The kinetic parameters were determined by fitting a progress curve assuming steady state Michaelis-Menton kinetics. The parameters of different substrates, each with a different P1 residue, can be compared to discriminate changes in the relative substrate affinity for different P1 side chains as well as changes in the acylation rate for different subtilisin variants at position 166. The results comparing the kinetic parameters of native subtilisin with variants having asparagine or lysine at position 166 are presented in Table II.

We find that while asparagine at 166 results in a variant subtilisin that under the conditions in Table I appears to be a more efficient enzyme, this efficiency is not coupled to a dramatic alteration in the acylation rate or in the dissociation constant

Table I. Relative Caseinolytic Activity of Codon 166 Variants

Codon 166	Activity Relative to wild type*
Asn	126%
Gly (wild type)	100%
Met	88%
Cys	88%
Glu	86%
Ala	84%
Tyr	81%
Pro	79%
Thr	79%
Val	78%
Arg	72%
Asp	68%
His	68%
Ser	60%
Gln	55%
Lys	44%
Trp	43%
Phe	42%
Leu	36%
Ile	18%

*Activity was normalized for protein concentration of electrophoretically pure material on the basis of absorbance at 280 nm. All reactions were stopped by precipitation with 10% trichloroacetic acid (TCA) after 20 min in 50 mM Tris-HCl, pH 8.0, 1% w/v casein. Activity was determined from absorbance units of the supernatant after precipitation with 10% TCA.

for the substrates measured. The one exception, common to all non-glycine 166 variants, is an increase in K_m for substrates with tyrosine at the P1 position (5).

The lysine 166 variant has quite different kinetic properties. In contrast to the caseinolytic results, the lysine 166 variant has comparable or higher efficiency on small peptide substrates compared to the native enzyme. Furthermore, the lysine 166 variant manifests a dramatic decrease in the dissociation constant for a substrate having a glutamic acid at the P1 position. The apparent acylation rate is unchanged, and as a consequence, the measure of catalytic efficiency, k_{cat}/K_m, is dramatically increased. Of equal interest is that the k_{cat} and K_m for large hydrophobic residue at P1 change very little and in some cases k_{cat}/K_m increases. In addition, the lysine 166 variant is 500-fold more efficient in the hydrolysis of the peptide bond following glutamic acid. The result for the lysine 166 variant is a very diverse enzyme that is highly efficient toward hydrolysis of the peptide bond following both glutamic acid and hydrophobic residues.

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Table II. Kinetic Constants for Specific Subtilisin Variants at Codon 166

Codon 166	Substrate*	k_{cat}	K_m	k_{cat}/K_m
Gly	sAAPFpNA	50.0	1.40×10^{-4}	3.57×10^{5}
Asn	sAAPFpNA	27.9	1.53×10^{-4}	1.82×10^{5}
Lys	sAAPFpNA	20.9	3.65×10^{-5}	5.73×10^{5}
Gly	sAAPLpNA	28.3	2.70×10^{-4}	1.05×10^{5}
Asn	sAAPLpNA	59.0	6.10×10^{-4}	9.60×10^{4}
Lys	sAAPLpNA	70.0	1.70×10^{-4}	4.10×10^{5}
Gly	sAAPApNA	2.03	1.48×10^{-4}	1.38×10^{4}
Asn	sAAPApNA	2.9	2.40×10^{-4}	1.20×10^{4}
Lys	sAAPApNA	2.2	8.10×10^{-5}	2.80×10^{4}
Gly	sAAPEpNA	0.54	3.40×10^{-2}	1.60×10
Asn	sAAPEpNA	0.25	6.03×10^{-3}	4.15×10
Lys	sAAPEpNA	0.69	5.60×10^{-5}	1.23×10^{4}

*Substrates sAAP(x)pNA have the form succinyl-Ala-Ala-Pro-(x)-p-nitroanilide where x designates the one letter code of the P1 amino acid (A-Ala, E-Glu, F-Phe, and L-Leu).

X-ray Crystallography

Crystals of the asparagine 166 and lysine 166 variants that are isomorphous with the crystal of the native enzyme have been grown. Diffraction data were collected to 2.25 and 2.0 A resolution and three-dimensional models have been determined and refined to a crystallographic R-factor of 0.16 and 0.17 for the lysine and asparagine 166 variants, respectively.

The availability of isomorphous crystal data allows for the direct examination of structural differences between variant and native enzymes in difference electron density maps, thus highlighting the changes that have occurred in the three-dimensional structure. The difference electron density maps between the 166 variant structures and the wild type enzyme showed no density outside the region of the substitution, indicating no significant conformational changes occur as a result of substituting either lysine or asparagine for glycine. Electron density for the substituted side chain along with small perturbations, indicating that some local rearrangement had occurred, were present in these difference maps.

In the case of the asparagine 166 variant, the electron density for the substituted side chain was complete (Figure 2), indicating that the side chain has a highly preferred conformation. The ND2 group of the asparagine 166 side chain is in position to hydrogen bond with the carboxyl of glutamic acid 156 which adopts a different conformation relative to the native enzyme. In the refined native structure this glutamic acid has a high temperature

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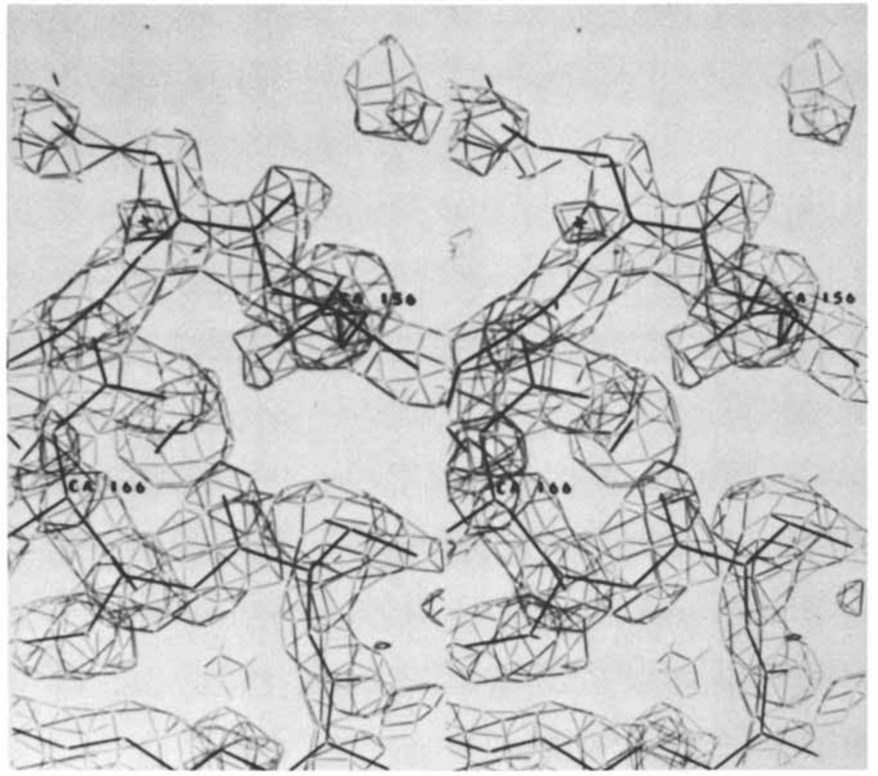

Figure 2. Stereoscopic view of the electron density map (2 Fo - Fc) of the asn 166 variant. The side chain density of asn 166 and glu 156 are complete and suggest that a hydrogen bond is formed.

factor. The temperature factor is significantly lower for glutamic acid 156 in the refined structure of the asparagine 166 variant suggesting that the hydrogen bond between the side chain of asparagine 166 apparently contributes to the reduction in the thermal parameters of 156 side chain. The 156 side chain is on the surface where the enzyme and protein substrates interact and the higher apparent activity of the asparagine 166 variant may result from the tethering of glutamic acid away from the site of interaction. Such an effect would explain the increase in activity for larger protein substrates even though the acylation rate and dissociation constants for peptide substrates remain unchanged.

The electron density map for the lysine 166 variant contains density for the lysine extended chain conformation (Figure 3). This density is weak at the extremity of the side chain indicating disorder, although the extended conformation predominates. The difference electron density map between the lysine 166 variant and the native enzyme shows density for part of the side chain (from the main chain through the gamma carbon atom). This is further evidence that there is disorder in the lysine 166 side chain. In the extended conformation the aliphatic carbon atoms of the side chain would have the effect of closing off the S1 site and could form favorable hydrophobic contacts with substrates with phenylalanine or leucine at the P1 position. However, the side chain can adopt alternate conformations, one of these can be modeled to form a hydrogen bond with a glutamic acid at the P1 position (Figure 4). This conformation would bring a positive charge into the S1 site. It is an attractive hypothesis that in the case of lysine 166, a glutamic acid might induce this conformation of the lysine side chain by a similar mechanism to that postulated by Koshland (13). This conformational variability can account for the dramatic increase in the affinity of the lysine 166 variant for a glutamic acid at P1 while not disfavoring the binding of hydrophobic residues such as phenylalanine or leucine at P1.

Conclusion

We have shown that single amino acid substitutions at position 166 have dramatic effects on the overall enzymatic activity and substrate specificity of subtilisin. In both the examples discussed, asparagine and lysine 166, the effects are linked to residues having some degree of conformational variability or disorder. Glutamic acid 156 becomes more ordered in the presence of an asparagine at position 166, and the side chain of lysine is capable of adopting conformations favoring a glutamic acid or hydrophobic residues at P1.

In this site-directed mutagenesis study as well as others involving subtilisin (14) and other proteins (15,16), the substitution of one amino acid has, in general, resulted in only localized structural perturbations. These changes, often subtle (rms shifts of less than 0.5 of an Angstrom), result in dramatic changes in activity and specificity. Localization of structural changes to the site of mutagenesis offers the potential to develop a database leading to algorithms predicting the structural consequences of any particular single amino acid substitution.

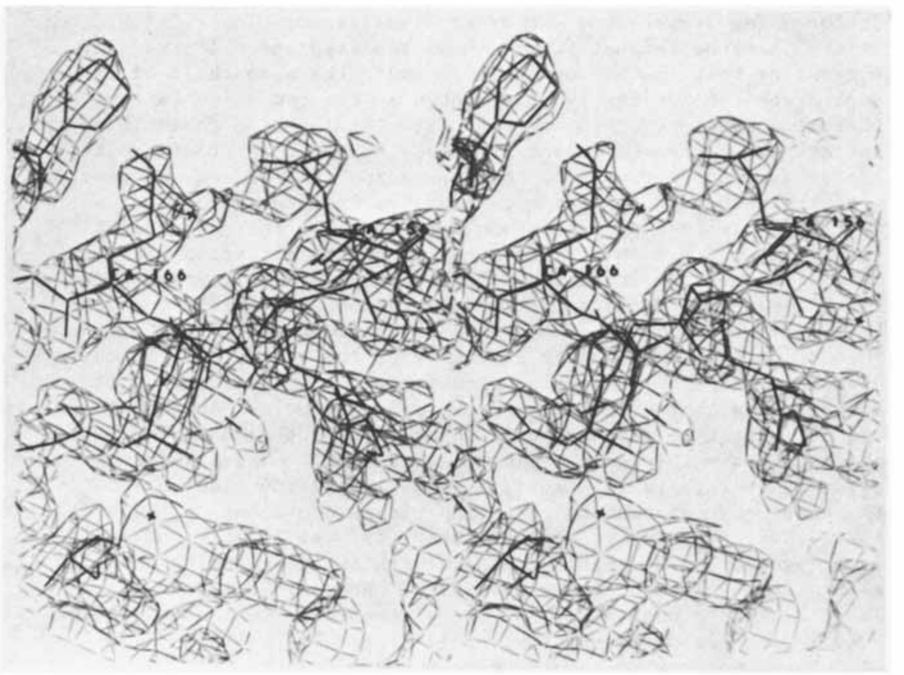

Figure 3. Stereoscopic view of the electron density map (2 Fo - Fc) of the lys 166 variant. The side chain density of glu 156 is discontinuous, while the density of the lys 166 side chain is weak. This indicates the side chains of the glu 156 and lys 166 adopt more than one conformation.

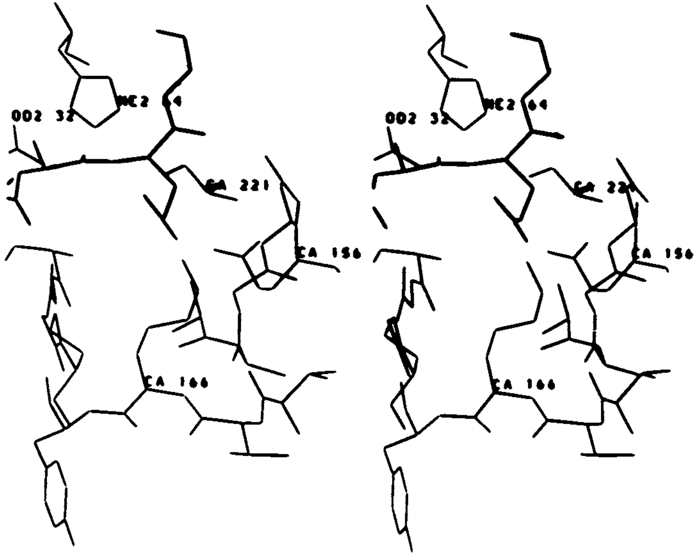

Figure 4. Stereoscopic view showing a model of the enzyme substrate complex for the variant with lysine at position 166 and a substrate having glutamic acid in the P1 position

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Reliable predictive algorithms should in turn speed the analysis of functional results of site specific mutagenesis.

One cautionary note raised by these results is that care should be taken to avoid over-generalization from a single mutant study. Arguing on the basis of higher relative activity on casein of the asparagine 166 variant, it would seem that tethering the glutamic acid 156 side chain is advantageous. On the other hand, the lysine 166 kinetic results argue that in this particular case, conformational variability increases the range of action. Although the lysine 166 variant has lower activity against casein, the experiment has the limitation of a single protein concentration and a single substrate concentration. A more reliable estimate is the ratio of k_{cat}/K_m. While it is clear that more data is needed to evaluate the relative importance of conformational variability on enzymatic efficiency, these examples suggest that protein engineering of this and other properties is a realistic goal.

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Chapter 12

Detection of Plasmids in the Plant Pathogenic Bacterium *Xanthomonas campestris* Pathovar *glycines*

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Fourteen of 16 available pathogenic strains of Xanthomonas campestris pv. glycines, obtained from geographically disperse locations, contain a plasmid of molecular mass 18 megadaltons (Mdal). The remaining two strains contain plasmids of either 11 or 29 Mdal which may be homologous to the 18 Mdal plasmid. All four non-pathogenic isolates of X. campestris pv. glycines are plasmid-free. The pathogenic strains harbor from 1 to 3 plasmids. With but a single 110 Mdal exception, all the plasmids are less than 30 Mdal in size. Nine of the pathogenic strains have in common an approximately 1 Mdal plasmid. The wide distribution of the 18 Mdal plasmid among the pathogenic isolates, and its absence from the non-pathogenic ones, suggests that it is required for pathogenicity.

Bacteria of the genus Xanthomonas are yellow-pigmented, slime-forming, motile, Gram-negative rods which infect and cause disease in plants. Within each species of the xanthomonads, individual isolates are differentiated into "pathovars" (pv.) depending on the identities of the plants which they infect. Xanthomonas campestris pv. glycines (X. c. pv. glycines) infects soybeans, causing bacterial pustule disease. Disease is characterized by localized hypertrophy of the plant cells, leading to the formation of small tumor-like masses (pustules) surrounded by yellow chlorotic haloes, primarily on the undersides of infected leaves. X. c. pv. glycines is capable of causing an 8-10% reduction in productivity upon infection of susceptible plants. The impact of this organism is greatly reduced, however, by the widespread use of soybean cultivars carrying a recessive gene pair, rxp (1), which confers resistance to bacterial pustule disease.

In bacteria, genes encoding functions which are not absolutely required for viability are often located on extrachromosomal DNA molecules (plasmids). Phytopathogenicity would seem to be such a dispensible trait and it is interesting to inquire as to the location of the genes conferring this property. Plasmids have been identified in some, but not all, plant pathogenic bacteria

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(2), including Pseudomonas solanacearum (3), various pathovars of Pseudomonas syringae (4-6), and X. campestris pv. vesicatoria (7). In none of these instances do the genes for pathogenicity appear to be plasmid-encoded. In contrast, plasmid-borne genes are required for the induction of crown gall disease in dicotyledenous plants by Agrobacterium tumefaciens (8,9) and in the production of oleander knot disease in oleander plants by P. syringae pv. savastanoi (10). The present report describes the results of our investigation of X. c. pv. glycines, both pathogenic and non-pathogenic isolates, for the presence of plasmids. In addition to providing fundamental information concerning the presence of plasmids in this organism, such studies can also provide information relating to the role of plasmids in pathogenesis. The presence of a particular plasmid in all pathogenic strains, and its absence from non-pathogenic isolates would indicate that some or all of the genes required for phytopathogenicity may be plasmid-encoded. Further characterization of such a plasmid might allow the location and identification of genes necessary for pathogenesis.

Materials and Methods

The origins of the X. c. pv. glycines examined in this study are listed in Table 1. One non-pathogenic strain (S-9-8) was pathogenic when first isolated from an infected soybean plant in the field but became nonpathogenic during culture on agar media. The other three non-pathogens were obtained from an established culture collection, and were unable to incite disease when revived from the lyophilized state. Strains were grown on an agar medium (11) and stored at 4°, with monthly transfer to fresh media. Cell lysates were prepared by alkaline detergent lysis of cultures grown overnight in L broth (11). After the addition of lysing solution the samples were heated sixty minutes at 57°. This effected the removal of chromosomal DNA from the lysates. Aliquots (100 µl) of the lysates were electrophoresed in horizontal 0.6% agarose gels (11) which were then stained with ethidium bromide (12). Plasmids were visualized by illumination of the stained gels with ultraviolet light.

Bacterial strains bearing plasmids of known sizes, to serve as molecular weight markers during electrophoresis, were obtained from Drs. P. Walsh and G. Jacoby. Lysates of these were prepared by the method described above.

Results

Cell-free lysates prepared from 16 pathogenic and four non-pathogenic strains of X. c. pv. glycines were examined for the presence of plasmids by agarose gel electrophoresis. No plasmids were detected in the four non-pathogenic strains examined (Figure 1, left-most four lanes). All pathogenic strains contain one to three plasmids (Figure 1). Fourteen of the 16 pathogenic strains contain a plasmid of approximately the same size (Figure 1).

Lysates from selected strains were electrophoresed along with lysates of bacteria containing plasmids of known molecular mass (Figure 2). A graph relating the electrophoretic mobilities of

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TABLE 1: ORIGINS AND PLASMID COMPLEMENTS OF THE *XANTHOMONAS CAMPESTRIS* PV. *GLYCINES* INVESTIGATED

STRAIN	SOURCE	ORIGIN		PATHOGENCITY	PLASMIDS (MDAL)
J3-27-1A	W. FETT	WISCONSIN,	USA.	+	18
S-9-4	"	"	"	+	18
R-12	J. DUNLEAVY	IOWA	"	+	1.0, 17
MINN	B. KENNEDY	MINNESOTA	"	+	11
XP29	M. STARR	KENTUCKY	"	+	19
XP144	"	INDIANA	"	+	18, 26
17915	ATCC[1]	ILLINOIS	"	+	29,110
B83	L. FERRIERA	BRAZIL		+	1.2, 19
B93	"	"		+	1.2, 18
B97	"	"		+	1.2, 18
B99	"	"		+	1.2, 18
1124	NCPPB[1]	ZAMBIA		+	1.2, 18, 26
XP202	M. STARR	"		+	1.2, 18
1714	NCPPB	"		+	1.2, 17
1717	"	ZIMBABWE		+	18
XP175	M. STARR	SUDAN		+	1.2, 18
S-9-8	W. FETT	WISCONSIN,	USA	-	NONE DETECTED
1136	NCPPB	ZIMBABWE		-	" "
1716	"	ZAMBIA		-	" "
1135	"	ZAMBIA		-	" "

[1]ATCC: AMERICAN TYPE CULTURE COLLECTION, NCPPB: NATIONAL COLLECTION OF PLANT PATHOGENIC BACTERIA.

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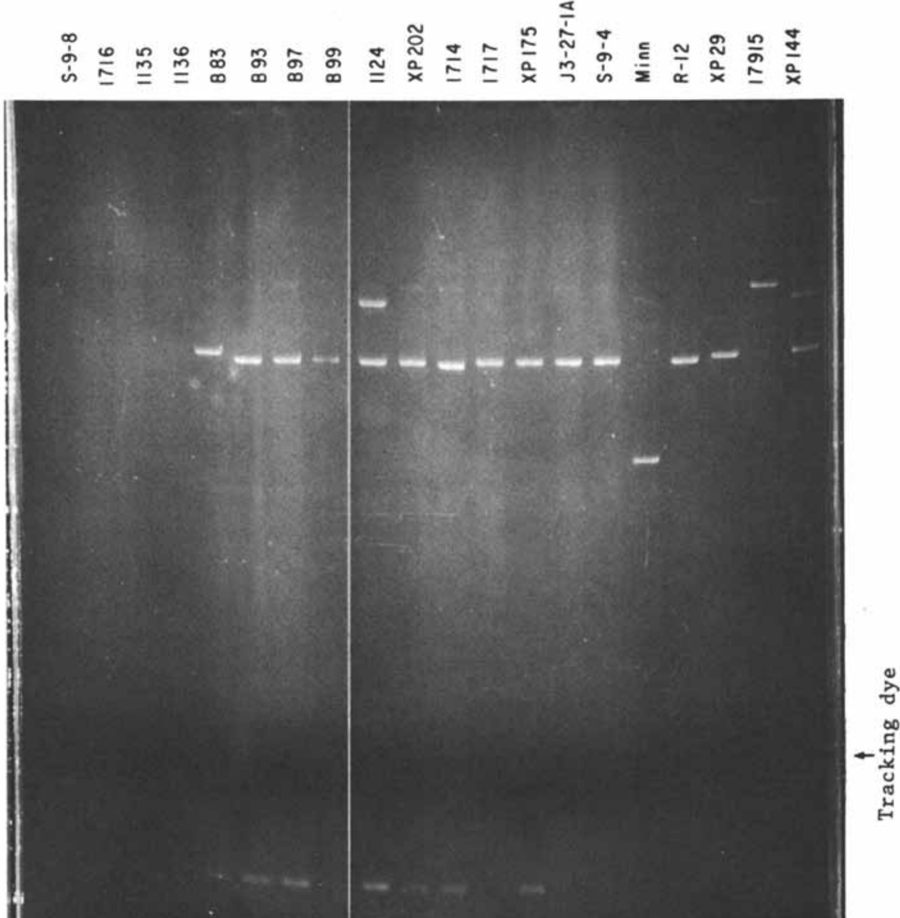

FIGURE 1: Electrophoretic analysis of cell lysates of X. campestris pv. glycines for the presence of plasmids. Lysates were loaded into the wells visible at the top of the figure and electrophoresed into the gel (downward direction in the figure) as described in the text. The identities of the lysates are listed along the top of the figure. The four left-most lanes bear lysates of the non-pathogenic strains.

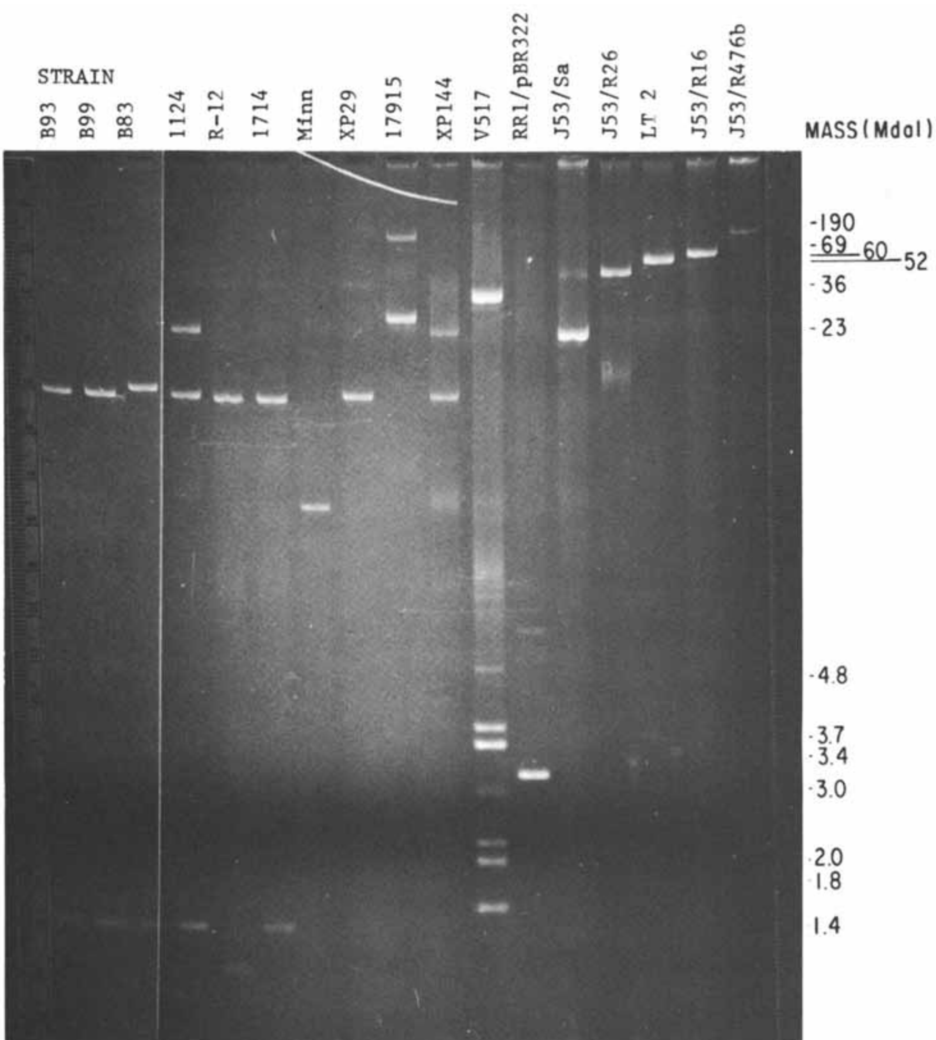

FIGURE 2. Electrophoretic determination of the molecular weights of the plasmids of X. campestris pv. glycines. Lysates were applied and electrophoresed as described in Figure 1. The 10 lanes on the left of the figure contain samples from selected X. c. pv. glycines. The rightmost lanes harbor lysates from strains containing plasmids of known molecular mass. Molecular weights of these standards are listed along the right side of the figure. V517: E. coli V517, harboring 8 plasmids of masses 1.4, 1.8, 2.0, 3.0, 3.4, 3.7, 4.8 and 36 Mdal; RR1/pBR 322: E. coli RR1 containing plasmid pBR 322 (3 Mdal); J53/Sa: E. coli J53 harboring plasmid Sa (23 Mdal); J53/R26: E. coli J53 bearing plasmid R26 (52 Mdal); LT2: Salmonella typhimurium LT2, which contains a 60 Mdal plasmid; J53/R16: E. coli J53 containing the plasmid R16 (69 Mdal); J53 R476b: E. coli J53 harboring the 190 Mdal plasmid R476b.

the standard plasmids to the logarithms of their molecular weights was constructed and used to estimate the molecular weights of the X. c. pv. glycines plasmids (Table 1). The X. c. pv. glycines plasmids range in size from 1.0 to approximately 110 megadaltons (Mdal). Most are less than 30 Mdal, with only one plasmid (in strain 17915) exceeding this value. The similar-sized plasmid present in 14 of 16 pathogenic strains has a molecular weight of approximately 18 Mdal. There appears to be some variation in the size of this plasmid from strain to strain. In some isolates (XP29, B83) it appears to be 19 Mdal, while in others (R12,1714) it is 17 Mdal in size. Two strains lack a plasmid in this size class. Strain Minn possesses instead a smaller, (11 Mdal) plasmid and strain 17915 harbors a 29 Mdal plasmid.

Nine of the strains contain a small plasmid. In eight instances it is 1.2 Mdal, while in the remaining isolate it is 1.0 Mdal. Twenty-six megadalton plasmids are contained in XP144 and 1124, in addition to the 18 Mdal and the 1.2 and 18 Mdal plasmids found, respectively, in these strains.

Conclusions and Discussion

An understanding of the biochemical and molecular genetic bases of bacterial phytopathogenicity may allow the development of means to control plant disease. Since disease often involves an alteration of the metabolism of the host plant, it is also possible that characterization of the mechanisms by which pathogenesis is induced may facilitate a rational approach to the directed modification of plant metabolism in order to increase productivity or agricultural utility. These studies were conducted as part of a program to characterize the genetic basis of the bacterial pustule disease induced in soybeans by X. c. pv. glycines.

No plasmids were detected in the four non-pathogenic strains examined. The fact that the lysis method employed here is capable of detecting plasmids at least as large as 190 Mdal (Figure 2) indicates that no plasmids below this size are found in the non-pathogens.

All 16 pathogenic isolates contain plasmids. Most harbor only one or two, and these are relatively small, exceeding 30 Mdal in only one case. Fourteen of the sixteen pathogenic strains contain a plasmid between 17 and 19 Mdal. Their similar sizes suggest that these plasmids are identical or very closely related. It is extremely unlikely that each of 14 individually isolated bacteria would harbor unrelated plasmids of essentially the same size. We therefore propose that the plasmids of approximately 18 Mdal are identical or very closely related.

Two of the pathogenic strains lack the 18 Mdal plasmid, and bear either a smaller (11 Mdal, strain Minn) or larger (29 Mdal, strain 17915) plasmid. Given the genetic plasticity of DNAs (13) it is reasonable to postulate that these arose from the 18 Mdal plasmid family by deletions or insertions. The small variations in the sizes of the 18 Mdal plasmid family from strain to strain may be the result of similar events. The presence of a similar-sized plasmid in the majority of the pathogenic strains, and the possible presence of homologous DNA sequences in the remaining strains,

suggests that some or all of the genes required for pathogenesis are plasmid-borne. This postulate is supported by the absence of the 18 Mdal plasmid from the non-pathogens. Experiments to further define the degree of homology between these plasmids, and to elucidate their roles in phytopathogenicity, are underway.

The 1.0 to 1.6 Mdal plasmids found in nine of the pathogens may also be related to one another. Since this plasmid is not found in all the pathogens it cannot be vital to the ability to cause disease. That it is found in pathogens obtained from geographically disperse locales (North and South America, Africa) suggests that it may confer some selective advantage upon cells harboring it, perhaps in a fashion related to pathogenicity such as by extending host range or enhancing virulence.

Acknowledgment

We gratefully acknowledge the excellent technical assistance of Deborah J. Fleming.

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Chapter 13

Use of Microorganisms and Microbial Systems in the Degradation of Pesticides

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Numerous agricultural chemicals have been shown to be degraded to varying degrees by microorganisms isolated from soil and water. By studying the biochemical and genetic basis of pesticide metabolism in bacteria we hope to be able to maximize the efficiency of these biochemical degradation processes so that feasible biological waste disposal methods can be developed. Hydrolase enzymes which degrade methyl-carbamate insecticides or organophosphorus insecticides have been isolated from several bacteria and have been partially characterized. One of these, parathion hydrolase, has proven useful in waste processing. The gene encoding parathion hydrolase has been shown to be identical in two very different bacteria. In both cases the gene is carried on plasmid DNA. While the genes are identical DNA-DNA hybridization and restriction enzyme mapping of the plasmid DNA have shown that the DNA outside the gene for parathion hydrolase differs markedly between the two bacteria. One bacterium which produces parathion hydrolase has proven useful in the elimination of the insecticide coumaphos, used extensively in cattle dipping operations.

The effective disposal of small volumes of aqueous pesticide wastes is one of the major practical problems facing American agriculture today. Public concern for the state of groundwater purity and the broad regulations embodied in the Resource Conservation and Recovery Act (RCRA) have stimulated renewed and increased interest in the use of microorganisms with unique biodegradative properties for the safe disposal of agrochemical wastes.

As demonstrated by the broad range of topics presented during the course of this symposium, biotechnology offers tremendous potential for significant advancements in many areas of agriculture. Our laboratory is investigating the role that biotechnology can play in the management of pesticide residues in the environment. One such

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role is in the elimination of pesticide residues in aqueous wastes generated after application and rinsing operations. Another is in removing residual pesticide from containers prior to their disposal. Our hope is that the techniques commonly associated with biotechnology will facilitate the development of efficient, low-cost biological methods that farmers or pesticide applicators can use on-site for effective waste management. Yet another area of active interest is in using biotechnology to control the rate of microbial degradation of a pesticide in the field so that an effective concentration of pesticide is maintained long enough to control target pests.

Pesticide contamination of groundwater may occur as a consequence of two types of situation. First, there may be problems resulting from the application of pesticides under field conditions for the control of active pests. Most soil applied pesticides exert their effects in the root zone. Once a pesticide escapes this zone it becomes a waste material. Movement of some compounds out of the root zone, through the vadose zone and into groundwater is of particular concern. In reality, only a fraction of a percentage of any pesticide applied to soil actually reaches groundwater (1). Nevertheless, with modern analytical capabilities, these small but detectable concentrations of pesticide residues are the basis for the present concern for groundwater quality. The second problem is the disposal of waste waters generated by farmers, commercial applicators, and industry from excess or unused aqueous pesticide solutions. If improperly handled, these wastes may also lead to groundwater pollution. This situation is especially acute where unlined waste disposal pits are used for disposal. Here, accumulation of pesticide residues may overwhelm the inherent binding and biodegradative capacity of the soils, leading to rapid migration of relatively large amounts of pesticide through the soil and into the groundwater.

There is a preponderance of evidence that most agricultural pesticides are subject to some degree of microbial metabolism. It is beyond the scope of this article to attempt to review the more than three decades of research on the role of microorganisms in pesticide metabolism. A number of excellent reviews on this subject are available (2,3,4,5). A major message that can be derived from past research is that there exists in the soil microbial community a wealth of genetic material that could be exploited for the controlled degradation of pesticides and their products in waste disposal efforts. We will describe our efforts to elucidate some of the biochemical and genetic mechanisms of pesticide metabolism by soil bacteria and to exploit biological degradation of pesticides in a waste disposal situation.

Biochemical Basis of Pesticide Degradation in Microorganisms

Numerous biochemical reactions directly affecting pesticides have been described using pure cultures of bacteria. Enzymes which catalyze the conversion of pesticides tend to fall into two classes; hydrolases (esterases, amidases, halidohydrolases) and oxygenases (mono or dioxygenases).

Hydrolases have a number of properties that make them attractive for use in waste disposal strategies. They require no cofactors for activity, are stable over a wide range of pH and temperature, and have a broad substrate specificity. Examples of previously described hydrolases affecting pesticides are the halidohydrolases which dehalogenate dalapon (2,2,-dichloropropionic acid) and a number of other halogenated aliphatic acids (6,7,8); the amidase described by Wallnofer et al (9,10) which rapidly degrades the acylanilide herbicide propanil and several urea herbicides (linuron, diuron); and parathion hydrolase (phosphotriesterase) (11,13) which degrades parathion and a number of related 0,0-diethylthiophosphorate insecticides. Munnecke (12,13) has demonstrated that the parathion hydrolase from Pseudomonas diminuta can be used effectively in the degradation of organophosphate insecticide wastes, ranging from industrial process wastewaters to residual material left in containers.

Oxygenases tend to be more complex enzymes and can be divided into two groups; mono-oxygenases (previously called mixed-function oxidases) which require reduced pyridine nucleotides as cofactors, and dioxygenases which do not require reduced compounds as cofactors. All oxygenases require molecular oxygen as a substrate, and tend to be less stable than hydrolases. An example of an oxygenase which directly affects a pesticide is the enzyme which is responsible for 2,4-D (2,4-dichlorophenoxyacetic acid) degradation in pure cultures of Alcaligenes and Arthrobacter. (14, 15). Presumably a similar enzyme is responsible for the first step in the degradation of 2,4,5-T (2,4,5-trichlorophenoxyacetic acid) by a pure culture of Pseudomonas cepacia (16). Although it is unlikely that oxygenase enzymes themselves will be useful in waste degradation systems, it has been demonstrated that the 2,4,5-T degrading P. cepacia can effectively remove the herbicide from heavily contaminated soils (17,18).

Recent reports have demonstrated that the white rot fungus Phanerochaete chrysosporium produces a peroxidase-like lignin degrading enzyme which can attack recalcitrant pesticides such as DDT and pesticide related compounds such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin) (19). The fungi certainly exhibit a broad range of degradative capabilities which deserve attention in future research efforts on biological elimination of pesticide wastes.

Using a modified enrichment technique, we isolated a bacterium which was very proficient in degrading the insecticide and nematocide carbofuran while utilizing it as a source of nitrogen (20). This organism is capable of rapidly degrading a number of other related N-methylcarbamate insecticides (Figure 1). We have isolated and partially purified an enzyme from this Achromobacter sp. which rapidly cleaves the N-methylcarbamate side chain of carbofuran (Figure 2) yielding the 7-phenol metabolite (2,3-dihydro-2-dimethyl-7-benzofuranol). This enzyme seems to fall into the hydrolase class of enzymes. It was active over a broad range of pH and temperature, and once partially purified it was relatively stable at 4C. Extensive dialysis of the enzyme did not affect its ability to cleave carbofuran, indicating that soluble

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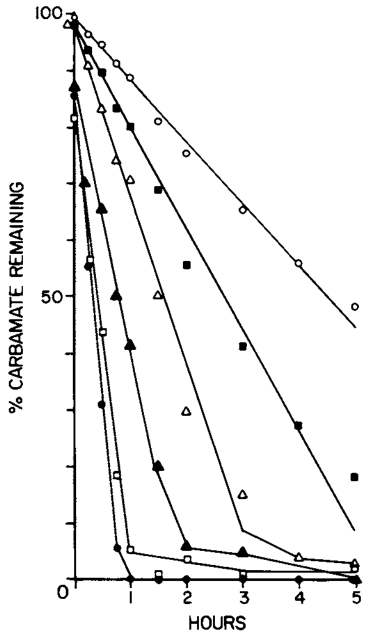

Figure 1. Hydrolysis of several N-methylcarbamate insecticides by resting cells of Achromobacter sp WM 111. Each compound was added to 100 ug/ml and they and their hydrolysis products were resolved by HPLC on a C18 column in a solvent of acetonitrile and phosphoric acid. Key: (■), aldicarb; (△), baygon; (●), carbofuran; (▲), carbaryl; (□), 3,5-dimethylphenyl-N-methyl carbamate; and (○), o-nitrophenyldimethylcarbamate. (Reproduced with permission from reference 20. Copyright 1986, Academic Press.)

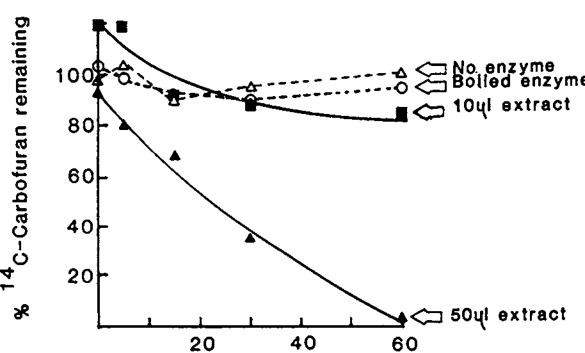

Figure 2. Carbonyl ^{14}C-labeled carbofuran was added to a reaction mixture containing varying amounts of a crude extract from Achromobacter sp WM 111. At various times samples were removed, acidified to drive off generated $^{14}CO_2$ and counted in a liquid scintillation counter.

cofactors were not required. This enzyme also hydrolyzed carbaryl, aldicarb and two related N-methylcarbamate insecticides.

Genetic Basis of Pesticide Degradation in Microorganisms

In bacteria, genes encoding unique biodegradative functions are often encoded on plasmids (21). Plasmids are discrete, autonomously replicating pieces of DNA that are distinct from the chromosome of the organism. They range in size from a few kilobases of DNA to several hundred kilobases. Many pesticide degradation genes are also plasmid encoded. Genes for the degradation of 2,4-D (22) and dalapon (23) have been shown to be encoded on plasmids. The 2,4,5-T degrading *P. cepacia* contains plasmid DNA but the role of this plasmid in encoding the degradation of 2,4,5-T is unclear at present. The carbofuran degrading *Achromobacter* sp also contains plasmid DNA (Figure 3). The plasmid in this strain is large (over 100 kilobases in size) and electrophoreses poorly as whole plasmid, but yields a finite number of bands upon digestion with restriction enzymes. The role of this plasmid in encoding carbofuran degradation is currently under investigation.

Parathion hydrolase has been detected in a number of different organisms (2). The gene encoding parathion hydrolase has been shown to be present on a 66 kilobase plasmid in an American isolate of *Pseudomonas diminuta* (32). The gene encoding this hydrolase has been cloned and introduced into various bacterial strains (33). Recently, our group has shown that the gene which encodes a parathion hydrolase in a *Flavobacterium* sp. (isolated in the Philippines (24)) was encoded on a 43 kilobase plasmid (25).

DNA-DNA hybridization experiments using the parathion hydrolase genes(termed *opd* for organophosphate degradation) from *P. diminuta* and *Flavobacterium* indicated that the genes from these two sources were very similar, if not identical. Restriction mapping of cloned DNA fragments from both organisms also suggested that the DNA encoding the *opd* gene itself is very similar in these two organisms. However, this mapping has also shown that the plasmid DNA outside of the *opd* coding region is very different in the two organisms (Figure 4). The observation that the parathion hydrolase in two temporally, geographically, and biologically distinct isolates of bacteria was encoded by identical genes carried on non-identical plasmids suggests that the gene may be part of a mobile genetic element or transposon (26).

Coumaphos: A model pesticide degradation system

The Animal and Plant Health Inspection service (APHIS) of the USDA in cooperation with the state of Texas carries out extensive cattle-dipping operations along the border of Texas and Mexico. The program is designed to prevent the reintroduction of the cattle fever tick (Boophilus) into the United States. The insecticide currently used in this operation is coumaphos [0,0-diethyl 0-(3-chloro-4-methyl-2-oxo-2H-1-benzopyran-7-yl) phosphorothioate]. Annually, this operation generates over 570,000 L of aqueous wastes containing 1500 to 3000 ug/ml of coumaphos. Currently this waste is placed in concrete lined evaporation pits. Since coumaphos has a very long

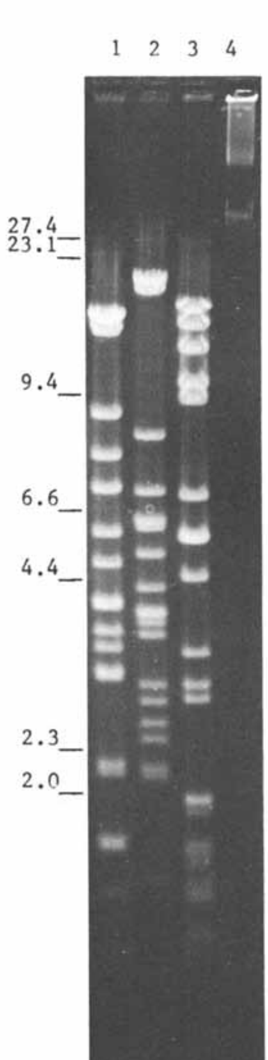

Figure 3. Agarose gel electrophoresis of DNA obtained from the carbofuran degrading Achromobacter sp. WM111. Lane 1 - Eco RI digested; Lane 2 - Bam HI digested; Lane 3 - Hind III digested; and Lane 4 - untreated. The numbers to the left indicate the migration distance of the fragments in a Hind III digest of phage lambda included in the same gel and indicate the size of the fragments in kilobase pairs.

half-life (~ 300 days) in soil and water (27), a disposal method which assures the safe and effective conversion of coumaphos to less environmentally harmful products was sought.

The process of UV-ozonation, whereby aqueous pesticide suspensions are pretreated with intense ultra-violet radiation in the presence of oxygen prior to soil disposal, was shown to be effective in accelerating the degradation of several commonly used herbicides (28). Coumaphos was resistant to any degradation by UV-ozonation, with only 25% degraded over a 5 h period (29). Exposure of coumaphos cattle-dip suspensions to large concentrations of mechanically generated ozone indicated that this compound was very resistant to oxidation by ozone.

The parathion hydrolase enzyme produced by Flavobacterium sp ATCC 27551 was able to rapidly hydrolyze coumaphos yielding chlorferon (3-chloro-4-methyl-7-hydroxycoumarin) and diethylthiophosphoric acid as products (Figure 5). Resting cell suspensions of the Flavobacterium were very effective in degrading coumaphos (Figure 6A). These small scale experiments used a dense suspension of cells (10^9 cells/ml) to accomplish the degradation of the coumaphos within a short period of time. This practice may be impractical in the actual use of organisms for processing large volumes of waste.

The chlorferon produced as a result of the microbial hydrolysis of coumaphos was very susceptible to further degradation by UV-ozonation (Figure 6B). The entire combined process of microbial hydrolysis followed by UV-ozonation required less than 7 hours to affect the safe destruction of coumaphos. Although the chlorferon had completely disappeared after this process, most of the ^{14}C label originally present in the benzene moeity of coumaphos was still present in aqueous solution (Figure 6B). Gas chromatography/mass spectrometry of the organic products in solution has indicated that 2,4-dihydroxyacetophenone and short chain alkanoic acids are detectable products. These more polar organics were very susceptible to complete degradation by soil microorganisms (Figure 7). In contrast, neither coumaphos nor chlorferon were appreciably degraded by indigenous soil microorganisms. Moreover, coumaphos subjected to UV-ozonation without prior microbial hydrolysis underwent very limited metabolism.

The UV-ozonation process rapidly killed the Flavobacterium cells that were added to the dip-vat waste. This was expected since many European communities use UV-ozonation or direct ozonation to treat drinking water (instead of chlorination) for the control of harmful microbes. Thus, the end result of this process is an aqueous product that contains only readily biodegradable organic material.

This process was field-tested on 2470 L of coumaphos cattle-dip waste at the APHIS vats in Laredo, Texas. To overcome the need to deliver an extremely large number of Flavobacterium cells to the site, the organisms were added as a 1% inoculum (22.8 L of culture grown in nutrient broth plus xylose) along with 9.5 kg of xylose as a carbon source and 4.5 kg of ammonium sulfate fertilizer as a nitrogen source, in order to allow growth of the organisms in the waste. The pH of the material in the tank was adjusted to between 6.8 and 7.0 by the addition of 1.4 kg of monobasic potassium phosphate and 1.8 kg of

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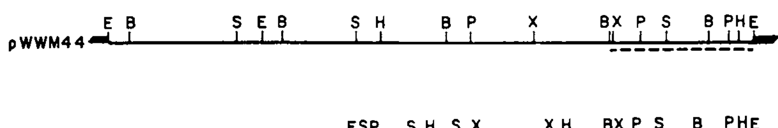

Figure 4. Restriction endonuclease maps of cloned Eco R1 fragments containing the opd genes from P. diminuta (from Serdar and Gibson [33]), and Flavobacterium sp. ATCC 27551. The thick lines represent the adjacent portions of vector DNA; the thin lines represent inserted plasmid DNA from Flavobacterium sp. (top) and P. diminuta (bottom). The underlined area delineates the 2.1 kilobase region of the two cloned fragments where the restriction maps are identical. Restriction endonucleases: B. Bam HI; E. Eco RI; H. Hind III; P. Pst I; S. Sal I; X. Xho I. (Reproduced with permission from reference 25. Copyright 1985, American Society for Microbiology.)

Coumaphos $\xrightarrow{H_2O}$ Chlorferon + $(C_2H_5O)_2P(S)OH$

Figure 5. Hydrolysis of coumaphos to yield chlorferon and diethylthiophosphoric acid as catalyzed by parathion hydrolase enzymes from Flavobacterium sp. ATCC 27551 and Pseudomonas diminuta.

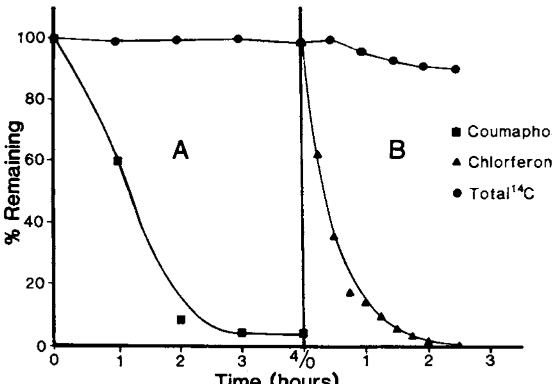

Figure 6. Spent coumaphos dip-vat solution was supplemented with U-Benzyl-^{14}C-coumaphos and *Flavobacterium* cells were added to a density of 10^9 cells/ml (Panel A). The hydrolysed material was then subjected to U.V.-ozonation (Panel B). Samples were taken at various times and ^{14}C remaining in the material was determined by liquid scintillation counting. Samples were diluted 1:10 with methanol and subjected to HPLC in a solvent system of methanol in phosphoric acid to determine coumaphos (A) and chlorferon (B) levels.

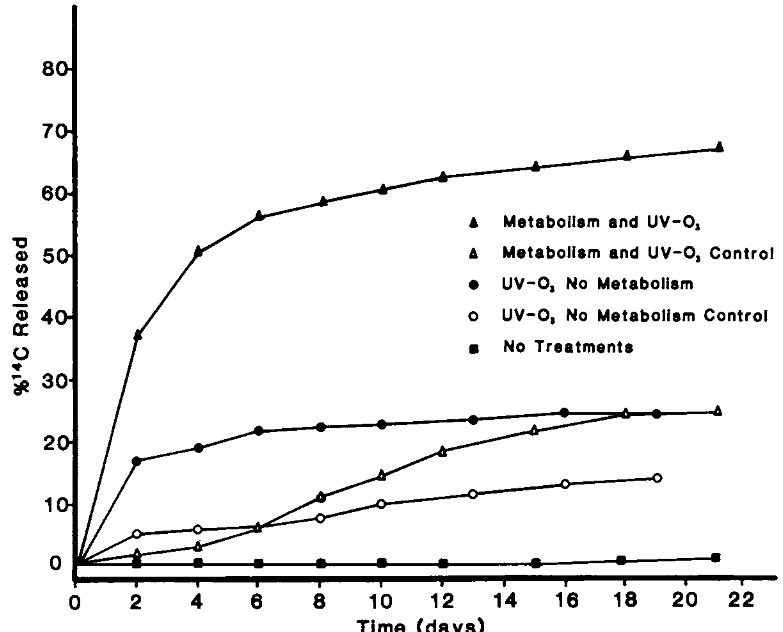

Figure 7. Soil metabolism of the products resulting from U.V.-ozonation of coumaphos and microbially hydrolyzed/U.V. ozonated coumaphos. $^{14}CO_2$ released from U-benzyl-^{14}C-coumaphos was measured in biometer flasks by trapping in potassium hydroxide and counting in a liquid scintillation counter. Controls contained soil that had been autoclaved one time but were not kept sterile.

dibasic potassium phosphate. The temperature of the liquid varied between 36°C and 33°C. The tank was vigorously aerated. Samples were taken at various times and diluted with methanol for later analysis of coumaphos and chlorferon. On-site determination of coumaphos levels in the tank was performed using a colorimetric assay kit (Bayvet, Shawnee, KS).

The microbial hydrolysis of coumaphos in the treated compartment was essentially complete within 48 hrs (Figure 8). As expected, the chlorferon hydrolysis product accumulated. At this point aeration of the tank was stopped and ozone (generated using a Griffin ozone generator) was introduced into the tank for about 20 hrs. Subsequent analyses indicated that over 50% of the chlorferon was degraded during ozonation. Laboratory analyses on smaller volumes of material had indicated that chlorferon was very susceptible to oxidation by mechanically generated ozone in the absence of U.V. light.

The results of the first Laredo field trial indicate that the method of microbial hydrolysis-ozonation was very effective in the elimination of waste coumaphos. A second, more extensive field trial is planned for the late spring of 1986. Among the items to be tested are alternative methods for the delivery of active cultures to a remote location. The delivery of adequate amounts of ozone into the hydrolyzed material was also a problem during the first field trial. However, this is strictly a problem of engineering existing technology to fit this unique purpose and should pose no great difficulty to the development of the overall process. As noted above, large scale U.V.-ozonation units for the treatment of extremely large volumes of drinking water already exist. The technology behind such systems should be directly applicable to our purposes.

Discussion and Prospects

Indigenous soil and water microbial populations contain many members which have the potential for carrying out the bioconversion of pesticide molecules to nontoxic products. The isolation of individual cultures capable of altering pesticides and the biochemical characterization of pesticide degrading enzymes are the first steps in developing biotechnology for the safe disposal of waste pesticides. The molecular characterization of the genes encoding pesticide degradation provide background information for future molecular genetic manipulation of these genes for use in waste disposal techniques. It also provides basic information on the origin, evolution, and transmission of pesticide degradative genes in microorganisms.

One recent agricultural phenomenon that may be elucidated by such basic research is the manner in which problem soils arise. In these soils, the microbial population degrades applied pesticides so rapidly that pesticide efficacy is lost (30). Presumably, the repeated application of a particular pesticide acts as an enrichment procedure, constantly selecting for increased numbers of the particular microorganisms that can metabolize the pesticide and derive some nutritional benefit from it. Several basic questions that need to be answered in order to understand this process. First, where do the genes that encode the pesticide degradation enzymes

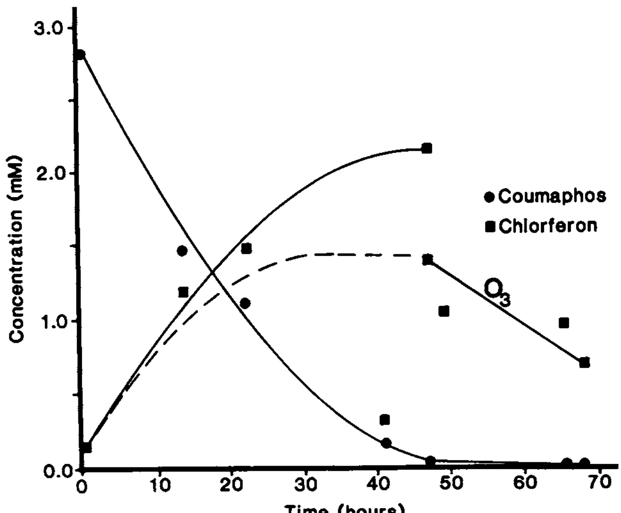

Figure 8. Metabolism and ozonation of coumaphos in a field trial on 2470 L of material in Laredo, Texas. Coumaphos and chlorferon measurements were made by HPLC as in legend to Figure 7. Erratic chlorferon measurements at 40h and 48h were due to precipitation of the chlorferon and settling out when aeration was stopped.

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originate? Second, are macromolecular events such as mutation, gene duplication, and gene movement between species necessary for the development of problem soils? The roles played by plasmids and transposons in the origin and propagation of pesticide degradation genes also need to be investigated.

As the coumaphos degradation process shows, biological pesticide degradation agents can be incorporated into viable waste disposal schemes. This particular process combines biological hydrolysis with chemical oxidation followed by chemical oxidation to accomplish the complete degradation of a pesticide molecule. The combination of single-step (or limited step) biological degradation with a physical/chemical process such as U.V. irradiation, U.V.-ozonation or ozonation can provide safe, easy, and cost effective degradation of extremely recalcitrant pesticides. The use of hydrolase enzymes such as parathion hydrolase or carbofuran hydrolase as a first step in waste elimination is particularly promising. These enzymes are active over a wide range of environmental conditions and the reactions they catalyze greatly diminish the acute toxicities of the insecticides. Thus, their use is convenient and can render waste safe to store until it can be further processed. This may be particularly important since the capital costs of devices such as U.V.-ozonators could be prohibitive for individual farmers. Hydrolyzed wastes could be stored until a cooperatively owned or privately rented processor could be brought around to finish the job. An additional safety factor incorporated into such a multistep process is that the organisms that produce these enzymes derive little or no nutritional benefit from the metabolism of the pesticide. Since they cannot grow on the pesticide they should not propagate in field situations where some pesticide "staying power" is required. Thus, use of such microorganisms does not threaten to create new problem soils situations.

Biotechnology offers the potential for many advances in pesticide waste disposal. Gene cloning techniques offer the methodology by which the genes encoding pesticide degradative genes can be moved into industrially useful microorganisms. Thus, important degradative enzymes can be produced in great quantities and provided at reasonable prices. Enzyme or organism preparations might be immobilized to create cartridge type digestors that can have long shelf-lives and long useful lives. Knowledge of gene sequences, protein structures and reaction mechanisms will enable researchers to use techniques such as site directed mutagenesis (31) to alter or increase the chemical substrate range of pesticide degrading enzymes. It is very clear, that if pursued vigorously, and utilized intelligently, biotechnology can offer viable solutions for the treatment of agricultural wastes and environmental hazards.

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Chapter 14

Biodegradation of *S*-Triazines: An Approach To Dispose of Recalcitrant Wastes

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Biological treatment of wastes, if available, is recognized as being less expensive and as producing a better-quality outflow than physico-chemical methods. Up till now, *s*-triazines (e.g., the herbicide atrazine) and by-products from chemical syntheses were regarded as non-biodegradable and wastes were usually treated physically. We have developed analytical HPLC methods to identify and determine routinely the whole range of by-products (e.g., *N*,*N*′-bis(ethyl)-*N*′′-(1-methylethyl)-1,3,5-triazine-2,4,6-triamine and 2-chloro-4-ethylamino-1,3,5-triazine-6(5*H*)-one) in wastes from these syntheses. We have also obtained aerobic cultures (e.g., *Pseudomonas* spp.) that quantitatively utilize the by-products as sole sources of nitrogen for growth. Biochemical pathways of catabolism have been elucidated and no toxic intermediates seem to be involved. A mixed culture has been used to treat real wastes from herbicide syntheses under non-sterile conditions and about 80 % conversion of *s*-triazines to cell material was observed. Small-scale (2 and 25 l) fluidized beds were constructed. The main problems encountered were the costs of the carbon source and oxygen and the low rates of some enzymes.

A need exists for the authentic treatment of wastes, whether due to our platonic respect for our fellow man and the rights of the next generation to a safe environment, or for legal reasons. I stress the word ′authentic′, i.e., real conversion of waste to something non-toxic, as a reminder that hiding wastes is a self-deception and a non-treatment which we or our children will have to cope with. There are good physical, chemical and biological treatments appropriate for different wastes, but biological treatment, where available, is less expensive than competing methods. Further, biological treatment is frequently needed for products resulting from physical and chemical treatments (1). So there is a sound economic reason for biological waste treatment.

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Activated sludge systems have been a mature technology for some 75 years (2) but many substances are not degraded in a sewage works. It may seem to be a contradiction, but our aim is the biodegradation of these 'non-biodegradable' compounds, which are also termed 'xenobiotic' or 'recalcitrant' compounds.

Xenobiotics are compounds that are foreign to the biosphere (see 3 for fuller discussion) and that, in contrast to natural products, are seldom degraded in nature. But xenobiotics have always been occurring in nature, say by the action of volcanoes, and these novelties are now considered to be natural products and are biodegradable. The problem nowadays is the enormous increase in the production and diversity of the chemical industry in about the last 40 years. Nature has not been able to keep up. So our work is really to direct natural processes to degradation of the compounds in which we are interested.

The critical aspect of this natural process, called biodegradation, is that it is enzymic, and enzymes catalyze specific reactions. So we have to work with specific xenobiotics and not with some undefined mixture. Thus we must have specific analytical chemistry available to eliminate the artifacts arising from using solely indirect assays.

Our probem is the xenobiotic waste (Table I) arising from the

Table I. Structures of *s*-triazines[a] and their abbreviations[b].

Abbreviation	Substitution at position		
	2	4	6
CCCT[c]	-Cl	-Cl	-Cl
CIET[c]	-Cl	$-NHCH(CH_3)CH_3$	$-NHCH_2CH_3$
IEET[d]	$-NHCH(CH_3)CH_3$	$-NHCH_2CH_3$	$-NHCH_2CH_3$
EEOT[d]	$-NHCH_2CH_3$	$-NHCH_2CH_3$	-OH
EEAT[d]	$-NHCH_2CH_3$	$-NHCH_2CH_3$	$-NH_2$
CEOT[d]	-Cl	$-NHCH_2CH_3$	-OH
EOOT[d]	$-NHCH_2CH_3$	-OH	-OH

[a] The *s*-triazines are symmetrical, six-membered rings with alternating carbon and nitrogen atoms: each carbon and nitrogen atom nominally contributes three bonds to the ring structure and the ring carries three substituents, one on each carbon atom (i. e., at positions 2, 4, and 6). [b] The four-letter abbreviation has one letter for each of the three ring substituents and the letter 'T' for the *s*-triazine ring. [c] The educt (CCCT) and the product, e.g., atrazine (CIET) do not occur in the wastes in significant amounts. [d] This is a major component of the waste and represents also possible homologues (e.g., EEET). Reproduced with permission from Ref. 33. Copyright 1986, John Wiley & Sons.

syntheses of the *s*-triazine herbicides, which are not degraded in sewage works (4) and for which no simple, single routine determination was available (5).

Analytical chemistry of s-triazines

The importance of methodology is seen in a review of work on the degradation of s-triazine herbicides (6); only 2 out of 12 sets of data are valid because only in those two cases was thorough analytical chemistry done. The intensive study of triazine metabolism and wastes became practicable with the advent of new methodology for the routine determination of these compounds. Several groups simultaneously developed similar methods to separate and quantify many s-triazines using reversed-phase HPLC columns and buffered mobile phases (5, 7): in the absence of buffer, separation can be seen to be poor (8, 9, 10). A low wavelength (e.g., 220 nm) is necessary for high sensitivity in a UV-detector, because the major peak in the UV-spectrum of s-triazines is usually in this region (5). Whereas the methods using acetonitrile to adjust the polarity of the mobile phase may offer better resolution (10), our use of methanol is based on grounds of lower toxicity and much lower price. Our method has proved itself in basic and applied research (6, 11, 12, 13, 14). To complement this routine use of analytical HPLC and semi-preparative reversed phase methods to obtain material for mass spectra (e.g., 6), identification of intermediates is aided by GLC of desalted (15), derivatized s-triazines (16, see also 17). The advent of mass spectrometry interfaced with HPLC (e.g., 18) should, if applicable to the mainly non-volatile intermediates, further simplify the identification of metabolites of s-triazines. An alternative HPLC method (amino phase) for some s-triazines is available (19) but it does not work well in our hands (12). And there is methodology for the products of s-triazine ring cleavage (20).

The first use of our assay was to confirm biodegradation of components of model waste by newly-isolated bacteria (6). The specific rates of degradation were sufficiently high that a practicable bacterial treatment of the wastes in a sensibly-sized reactor could be calculated. We observed that the analytical method functioned with waste water from ametryne production (12) and it was agreed that we should examine and treat the s-triazines (Table I) in the wastes from the production of atrazine (Fig. 1) and simazine.

Strategy of our biodegradation research

We chose to treat wastes directly as they emerged from the production unit and before they entered an activated sludge process. The waste triazines are thus at a high concentration and contained no other wastes: this simplified the process control and the analytical chemical evaluation of the treatment. We are thus proposing small biotreatment units specific for particular problem wastes; other groups propose to add new organisms to activated sludge plants (e.g., 21).

The problem was that the real wastes had little in common with the model wastes - the synthesis had been modified - and we could degrade only the wrong spectrum of compounds (13). So we had to restart the research programme (Table II) to obtain the organisms with the required degradative capabilities. The general approach to the microbiology, physiology and biochemistry of the project is sketched elsewhere (22, 23) and a detailed description is in preparation.

Table II. Recommended research strategy for the biodegradation of xenobiotic compounds

0) analytical methodology
1) enrichment cultures
2) growth physiology
3) biochemistry
4) engineering
5) evaluation

This method of learning how to approach a similar project correctly, causes a bias in the appraisal of the relative efforts required for the research. Of some 20 research years in the project (about half by doctoral students) 20 % of the time was for analytical chemistry, 70 % for microbiology, physiology and biochemistry, and 10 % for engineering. For this presentation of the waste treatment it is only necessary to know that the *s*-triazines are degraded quantitatively to NH_4^+ and CO_2 (and, where appropriate, to Cl^- and an unidentified alkyl group) via defined, converging pathways of hydrolytic reactions in which no toxic intermediates occur (6, 11, 24, 25, 26, 27). The *s*-triazines serve only as sources of nitrogen (25) so a carbon source must be added to allow growth.

Waste treatment

The wastewater from syntheses of chloro-*s*-triazine herbicides is a clear, colourless mixture of wastes from the chemical reaction and from washing the product. It has a total organic carbon content of about 1200 mg L^{-1}, a total Kjeldahl nitrogen concentration of about 810 mg of N L^{-1}, a biological oxygen demand after 5 days of about 530 mg of O_2 L^{-1}, and contains NaCl at about 35 g L^{-1}; the pH is about 12 (14).

The waste treatment is based on the biochemical activity of an aerobic mixed culture which is used under non-sterile conditions throughout the work. The culture consists almost entirely of bacteria and does not grow in the absence of a source of combined nitrogen. The wastes are essentially stable (13) and contain only *s*-triazines as a source of nitrogen (14).

Preliminary experiments (14) in batch culture used mineral growth medium containing a carbon source and real wastes as nitrogen source. The culture grows biphasically (Fig. 2), the higher specific rate (μ) being $\leq 0.2\ h^{-1}$ and the lower rate being $\leq 0.05\ h^{-1}$. The *s*-triazines disappear sequentially (Fig. 3) with EOOT (representing the behaviour of OOOT, EEAT and EOOT (see Table I)) being metabolized first, CEOT later, and IEET and EEOT (a catabolic product from IEET) slowly. The disappearance of *s*-triazines is about 75-80 %, a value that is confirmed by determinations of Kjeldahl nitrogen; the *s*-triazine-nitrogen is converted into cell material. The process has a temperature optimum of about 35-40 °C and the culture can tolerate up to about 50 % (v/v) wastewater. The wastes do not supply significant amounts of carbon for growth.

s-Triazine wastes can be treated in continuous culture (14) (not shown) and requirements for mineral components in the growth

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Cl
N N
iPrHN N NHEt

Fig. 1. The s-triazine herbicide atrazine. In the homologue, simazine, both aminoalkyl substituents are ethylamino; in the analogue, ametryne, the chloro substituent is replaced by the thiomethyl group.

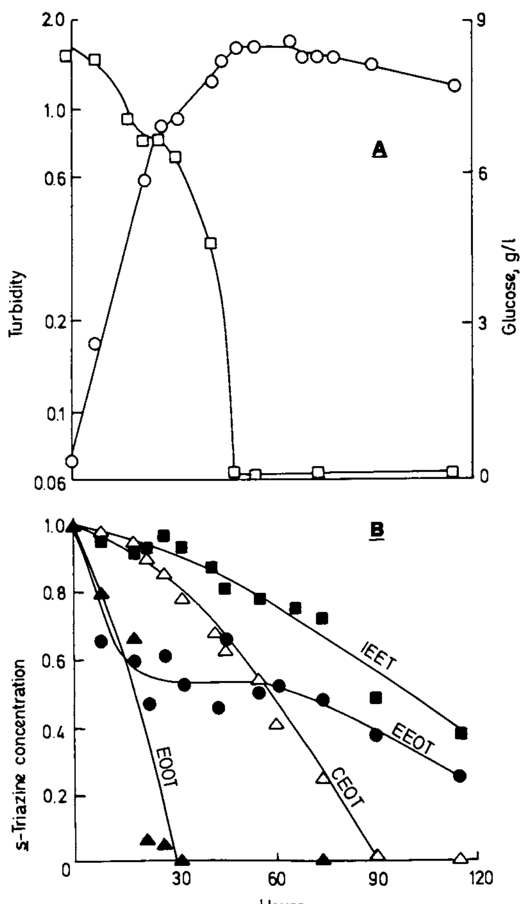

Figure 2. Growth and substrate utilization of the mixed culture in medium containing S-triazine wastes. Growth medium at pH 7.5 and 30 °C and containing 33% (v/v) wastewater and 10 g of glucose/L was inoculated from a continuous culture using wastewater. (A) Growth (○) was measured turbidimetrically. (B) Glucose concentrations (□) are absolute whereas the concentrations of S-triazines are plotted as a fraction of the initial values. (Reproduced with permission from reference 14. Copyright 1985, John Wiley & Sons.)

medium are met from components in tap water and in the wastes themselves. This simplified system is sensitive to excess carbon source, presumable to metabolites from the glucose used, and much fluctuation of biomass can be observed. Nevertheless, treatment efficiencies of 75-80 % are observed, but the dilution rate is low (0.025 h^{-1}). All components of the waste are degraded, but whereas e.g., EOOT disappears totally, IEET is only partially degraded unless the dilution rate is decreased.

The low volumetric loading rate possible in the continuous culture and the ease with which washout is induced, coupled with a requirement for a small unit, led us to seek a compact reactor that (a) yields high volumetric loading rates, (b) guarantees retention of the mixed culture, (c) provides a large surface area (per unit of reactor volume) for the attachment of cells, and, (d) shows less danger of clogging than a fixed bed reactor. Immobilized cell fluidized bed reactors (FBR) can meet this requirement (28), their application to waste treatment is known (29, 30, 31) and large units have been initiated industrially for waste disposal (32).

Each of our FBRs (2-L (Fig. 3) and 25 L) consists of a vertical column filled with carrier (quartz sand), on which most of the biological activity occurs, and a stirred tank for aeration of the culture (gas bubbles in the column disrupt the fluidization by causing the particles to float). The apparatus includes a thermostat, a pH-stat and regulation of the oxygen partial pressure by automatically supplmenting the aeration with O_2 as required. The recirculation rate through the fluidized bed is about 700-fold higher than the dilution rate of the reactor (33).

The mixed culture readily and spontaneously adheres to the sand particles while growing under batch conditions. When shifted to continuous culture conditions, the immobilized biomass stabilizes at 15-17 g/L after about a month. Excess biomass must then be removed to avoid inactive zones. This concentration of biomass is tenfold higher than observed in suspended culture and the specific growth rate is improved by about 50 % to 0.04 h^{-1}: this rate is about sevenfold lower than the value used to estimate the efficacy of cultures degrading model wastes (see above).

The degradative behaviour of cells immobilized in a FBR is shown in Fig. 4. All compounds are degraded, but not all to 100 %; about 75-80 % degradation is possible (closed symbols) and IEET (open symbols) is a good marker of general performance. This culture has an initial degradation rate of 1.6 mg of N/L.h and attains a maximum rate of about 26 mg of N/L.h (about days 33 and 50). That this is the maximum value is seen when the nitrogen concentration is further increased (day 40) and the system collapses. Recovery on reduction of the nitrogen concentration is rapid.

Several experiments are shown in Fig. 4. In days 13-20 the carbon input is at a reduced level and the efficiency drops; recovery is again rapid. An attempt to operate totally without a carbon source (day 23) leads to collapse of the system; the cells require 10-12 mol of C/mol of N. One major problem is masked in the performance data near day 40. Our supply of waste aged and a component (CEOT) hydrolyzed spontaneously to EOOT. On addition of fresh waste (day 40) the ability to degrade CEOT is missing and is restored by adding new cells. The observation of loss of degradation of CEOT is reproducible, so we must retain selective conditions in

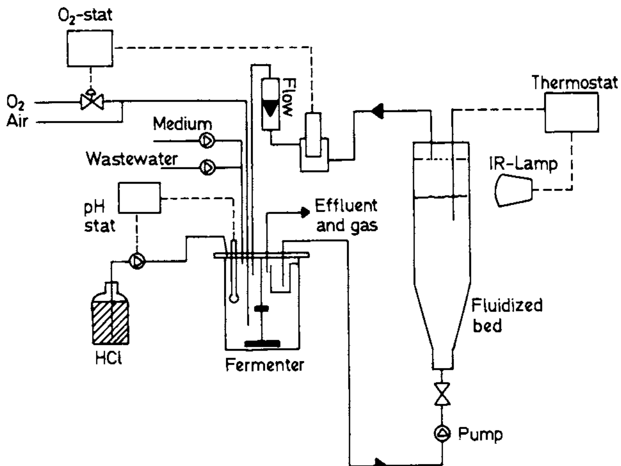

Fig. 3 Schematic representation of the small FBR. (Reproduced with permission from reference 23. Copyright 1985, Pergamon Press.)

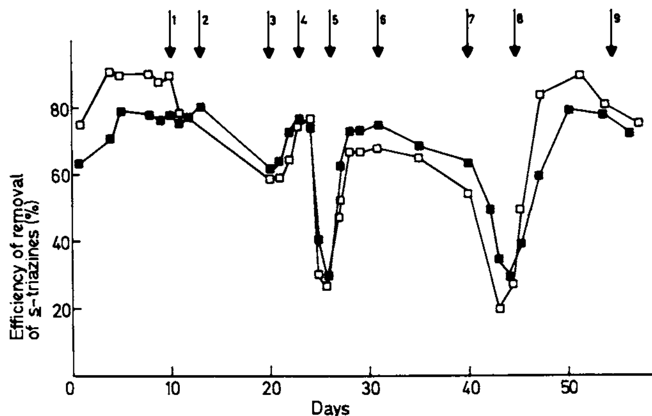

Fig. 4. Removal of dissolved s-triazines by the mixed culture in a continuous FBR reactor. The experiment was done at 30 °C and pH 7.5. At the numbered arrows, the following changes were made: 1) mean residence time reduced, nitrogen and carbon inputs increased; 2) carbon input reduced; 3) carbon input increased; 4) carbon input stopped; 5) carbon input restored; 6) mean residence time reduced, nitrogen and carbon inputs increased; 7) nitrogen input increased (new wastewater with higher nitrogen content); 8) nitrogen input corrected, mean residence time increased; 9) mean residence time reduced. Total s-triazine (■), IEET (□). Reproduced with permission from Ref. 33. Copyright 1986, John Wiley & Sons.

the reactor or risk losing activities. As in other cases, recovery is rapid, but this time due to addition of fresh biomass.

Evaluation of the project

An evaluation of the positive aspects of the project is quite simple. Straighforward analytical chemistry was developed and we enjoyed good cooperation with Ciba-Geigy to obtain, analyze and treat real wastes. The availability of the analyses not only proved the biology to be sound but also allowed the chemical engineers an alternative appraisal of the reactions in the syntheses. All the components of the wastes are now biodegradable - a complete change from a few years ago, when almost all of the compounds were non-biodegradable. Further, the degradative pathway contains no toxic intermediates to be excreted during a disruption of the system. And all the compounds can be degraded quantitatively. These are necessary preliminaries to applied work.

The development of a pre-pilot treatment unit has also been demonstrated, so it is possible to start with only the idea of biological treatment of recalcitrant waste and create a functional system.

The fact that the system is functional, however, does not mean that it is financially competitive with alternative methods. With hindsight we can note where the approach must be improved. This is seen first in the low degradation rates for some of the waste components. The chemical components in the real waste were identified late in the project and we had too little time to improve the degradation of these compounds. For this reason I again stress the need for analytical chemistry as the first step in biodegradation research, in order to allow time for the biology to be developed and quantified before committing oneself to engineering.

A second problem is that of the carbon source. As biologists we chose compounds which favoured our enrichment cultures, but which in practice are expensive. The factory complex, however, also has waste carbon that requires disposal, but it is not utilized by our strains. These two problems should have been combined from the initiation of the project to achieve integrated and thus economic waste disposal. Our initial idea to operate the treatment system for long periods under non-growing conditions (cf. 6) did not survive practical testing. Another property of the wastes, which was not considered in the enrichment cultures, was the salt concentration: if salt-tolerant organisms had been selected, the system could have been operated without dilution of the wastes.

A further source of expense is O_2, which must be supplemented to the fluidized bed reactor during the rapid aerobic growth with the wastes. This cost is inevitable with aerobic growth but would be eliminated in anaerobic growth. All reactions at the *s*-triazine ring and after its cleavage are hydrolytic, so anaerobic growth of appropriate organisms could also be anticipated with *s*-triazines as sources of nitrogen, and at least two *s*-triazines support anaerobic growth (11, 25).

It must be recognized that high biomass concentration alone is insufficient for adequate treatment. The biomass must contain the necessary enzymes, which can be lost under certain conditions, and

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it may be necessary to maintain reserves of representative wastewater or of, e.g., frozen active biomass in case there is an interuption in the production rhythm. A very delicate problem is the cooperation between the biologist and the engineer. The project will fail unless both workers are not only good but also respect one another.

This project reached its scientific goals but did not attain the commercially required reaction rate in our lab. Discussion of the reasons for the low rate shows them to be essentially trivial and that biodegradation of recalcitrant wastes is a feasable option in waste treatment (cf. 34, 35, 36).

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Chapter 15

Use of Microorganisms and Enzymes in the Synthesis and Production of Optically Active Agricultural Chemicals

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The production of both specialty and commodity chemicals by enzyme reaction has become a reality due to recent advances in immobilization. These immobilization techniques have provided an economical system for reuse of enzyme and thus provide a route to optical isomers in high enantiomeric yields. This provides specific stereoisomers for agricultural synthesis at reasonable cost. The advantages of stereoisomers include high activity levels as well as reduced toxicity due to the absence of the incorrect stereoisomer. Methods of immobilization will be reviewed with emphasis on immobilization by polyazetidine. Enzymatic reaction via immobilization enzymes and immobilized whole cells will be reviewed with emphasis on the production of agricultural chemicals.

The production of specialty chemicals by enzyme reaction has a high potential for having an impact upon agricultural chemical use, especially in the area of pesticides and herbicides. Since enzymes may be used to introduce chemical moieties in high yield and high enantiomeric excess at low temperatures and pressures, the use of enzymes as agents for introducing chirality into chemicals and producing chiral synthons will play an increasing role in agrichemicals. To date, mainly due to the expense of their production, chiral compounds have not been used extensively in agricultural chemicals. In fact, only two compounds are utilized commercially, Fusilade 2000 and the pyrethroids. Although agricultural chemicals may sell for significantly more than commodity chemicals, there are, nevertheless, limits to the sales price one can obtain when applying an herbicide or pesticide to a crop as opposed to the value of a lifesaving chemical which might be used in the production of

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pharmaceuticals. The pharmaceutical industry has, of course, used chiral compounds for decades. I believe that we are moving into an era in which agricultural chemicals will be based on compounds which have only one stereoisomer present.

Examples of currently marketed herbicides and fungicides which have a chiral carbon that might be responsible for increased activity are the herbicides Dual (metolachlor, Ciba-Geigy),1, Fusilade (Fluazifop-butyl,ICI),2, and Suffix bw (1 flam-prop isopropyl, Shell),3, and the fungicide, Ridomil(metalaxyl, Ciba-Geigy),4. All of these materials are sold as the racemic mixture;however, it is highly likely that one stereoisomer may have most of the activity for each of these compounds.

Moser, et al. have shown that of the 4 stereoisomers of metolachlor, the two having an S configuration of the chiral carbon atom have a stronger herbicidal effect.[1] Although metolachlor has only a weak fungicidal action, tests showed that the R configuration, in comparison to the S configuration, was 3 times more effective as a fungicide.[1] An effective method for preparation or for resolution and racemization of these compounds could be a potentially lucrative chemical goal. One can envision several points in the synthesis of metolachlor at which such a resolution might be carried out enzymatically.

The benefits of such a synthesis are numerous. In addition to lowered toxicity due to the lowered rate of application, one might also obtain lowered toxicity due to removal of the less active (or inactive) stereoisomer. Such a reduction occurred with the removal of the S isomer of thalidomide leaving all of the desired activity in the R isomer.[2] Had this fact been known at the time of the introduction of thalidomide in Britain, a great tragedy could have been avoided.

The main problem in the enzymatic synthesis of optically active agricultural compounds has been the lack of enzymes appropriate for these syntheses and/or resolutions and the lack of stability of such enzymes.

The advent of immobilized systems capable of producing chemicals in large quantity at considerably reduced prices provides an attractive route to chiral synthons for the agriculturally oriented organic chemist. Ideally, a chiral catalyst, whether biologically active or not, should have the following properties: excellent engineering characteristics, good stereochemical control, excellent flow properties, and long life-times, thus providing low cost.

Biological systems for catalysis have suffered in all these areas. Life-times for most systems have been notoriously short. Commercial systems for the production of high fructose corn syrup have had life-times on the order of 20-40 days. These immobilization systems have often been soft gels which have had problems in flow rate and in structural rigidity. The latter aspect complicates plant engineering due to the inability to stack these beads in large columns or to use high flow rates. The lack of stability results in increased labor costs due to catalyst changeovers in the columns. Plant productivity shows excessive swings in output due to the rapid

1

2

3

4

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decline of enzyme activity. In addition, there is an increased cost of enzyme or microbial cells used in production of the catalyst due to low stability.

Recently we have developed novel immobilization systems[3] which avoid these problems of lifetime, bead rigidity, flow rate and associated costs problems and thus allow the production of chiral synthons at considerably lower prices.

One example is the production of L-aspartic acid via immobilized aspartase contained in microbial cells. Thus, *E. coli* 11303 has been immobilized in a number of systems by various companies. (Table I)

TABLE I Commercially Developed Methods for the Production of Aspartic Acid

Immobilization Method	Activity μ moles/hr/gm cells (wet wt)	Retained Activity (%)	Half Life (Days at 37°)	Co.	Ref.
Polyacrylamide	18,850	49	120	Tanabe	4
K Carrageenan	56,000	56	70	Tanabe	5
Cross linked K Carrageenan (Hexamethylenediamine + Gluteraldehyde)	48,000	48	680	Tanabe	6
Polyurethane	68,000	100	37	Grace	7
Polyazetidine	68,000	100	1000-1400	PEI	8

Commercial production of pharmaceutical grade aspartic acid at levels of 4000 MT/yr has reduced selling prices to considerably less than $3.00 per kg, making it one of the cheapest amino acids available. The most economic method for production of aspartic acid is polyazetidine immobilization of *E. coli* 11303, based on the reaction given in Scheme 1.[7]

Thus, when polyazetidine prepolymer is mixed with *E. coli* 11303 and applied to a suitable support matrix (*e.g.* IRA 938 macroporous resin) and the polymer mixture cured by drying overnight in a gentle flow of 25% humidified air, a catalyst bead of approximately 0.3 mm diameter, which is quite strong and possesses excellent flow characteristics (up to 60 space velocities (S.V.)/hr. at less than 80 psi) can be generated. More than 97% of the aspartase activity of the free cell can be immobilized and retained. The half-life of this catalyst is estimated to be 1100-1400 days (Figure 1). A flow rate of 5.5 S.V./hr allows a 98.5% conversion of fumaric acid to L-aspartic acid with a final concentration of 19.8%. Based on this data, one can quickly calculate that a 200 liter column would produce in excess of 4 million lbs. of aspartic acid per year. Storage of the catalyst in 1.5 M ammonium

$$\bar{O}-\overset{O}{\overset{\|}{C}}-CH=CH-\overset{O}{\overset{\|}{C}}O-NH_4^{+} \longrightarrow {}^{-}O\overset{O}{\overset{\|}{C}}-CH_2-\underset{NH_2}{\overset{H}{C}}-\overset{O}{\overset{\|}{C}}O^{-}$$

SCHEME 1

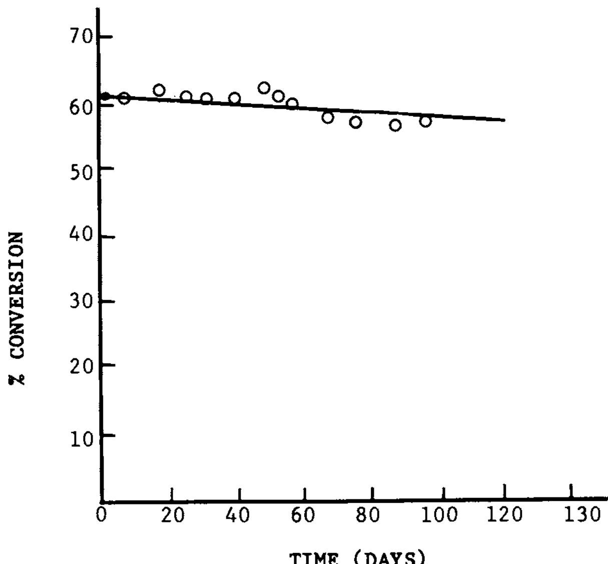

FIGURE 1 The stability of the aspartase catalyst was determined by continuously passing 1.5M fumaric acid, pH 8.5 (adjusted with NH_3), 1 mM $MgSO_4$ at 37°C at 6 volumes/volume/hr.

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aspartate at 4°C or at room temperature resulted in no decline in catalyst activity after a period of 4 years. Thus, the enzymatic approach to L-aspartic acid can provide a chiral synthon at extremely low prices.

It is of interest to note that metolachlor and metalaxyl can be considered as derivatives of a substituted amino acid, alanine. The most active form of metolachlor would correspond to an L-alanine derivative. L-alanine is a constituent of a number of biologically active compounds, among which are the pharmaceutical products Captopril (Squibb) and Alapril (Merck), both of which are angiotensin I converting enzyme inhibitors. Thus, L-alanine may be considered as a primary example of a chiral synthon of value in the production of both pharmaceutical and agricultural chemicals. The organic chemist will quickly realize that neither metolachlor nor metalaxyl is synthesized from L-alanine but rather their syntheses are based on substituted anilines. At the same time, it should be pointed out that L-alanine is not used in the synthesis of agricultural chemicals. I believe that the primary reason for this discrepancy is that, at the time of this writing, multi-ton quantities of L-alanine are quoted at $32-$45/kg. Based on these raw material costs, the use of L-alanine in the manufacture of most agricultural chemicals cannot be justified, even when a 50% reduction of raw material consumed is expected.

L-alanine can also be produced in a manner similiar to that for aspartic acid. The enzyme aspartate-β-decarboxylase, catalyzes the loss of CO_2 from aspartic acid, thus producing alanine. (Scheme 2)

The main problem encountered in this reaction is the CO_2 production, which is voluminous. Column reactions require pressure vessels[8] and gel type catalysts will not withstand the abrasion which occurs in a continuous stirred tank reactor or fluidized bed reactor[9]. _Pseudomonas dacunhae_ or _Alcaligenes faecalis_ have been immobilized by the polyazetidine method, thus providing a catalyst having high activity which is quite rigid. This catalyst can withstand the conditions of slow stirring in a continuous stirred tank reactor without degradation of the enzyme activity (Figure 2). The projected half-life, based on this data, is in excess of 6 months. Low cost alanine is a reality if large quantities (> 1000 MT) are required. In fact the price would be equivalent or below that of the chemically produced racemic mixture at that level.

In the area of pesticide chemistry the pyrethroids are an outstanding example of the use of chiral molecules on a commercial scale. Roussel-Uclaf has commercialized Decis, which is the d-cis-isomer of the pyrethroid, deltamethrin, **5**.[10] This insecticide is reported to have sales in excess of 500 million Franc in 1982. The total plant capacity is 225 metric tons per year. Decis is prepared by resolution of DL trans chrysanthemic acid. This resolution utilizes the base of chloramphenicol which forms a diastereomer that can easily be separated by filtration. Thus, chrysanthemic acid has been a chiral synthon which has been sought by a number of

$$H_2N-CH(CH_2COO^-)-COO^- \longrightarrow CH_3-CH(NH_2)-COOH + CO_2$$

SCHEME 2

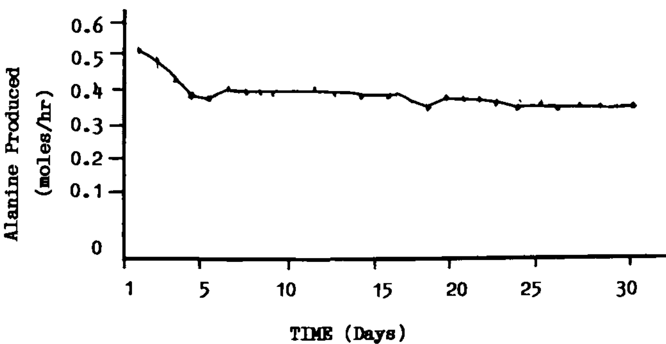

Figure 2 The stability of the immobilized organism containing aspartate-β-decarboxylase was assessed by continuous infusion of 1.5M aspartate (pH 8.5), 0.1 mM pyridoxal-5-phosphate, 0.5 mM sodium pyruvate at 37°C through a 750 ml volume continuous stirred reactor containing 75 mL of catalyst (0.2g <u>Pseudomonas dacunhae</u>/ml beads) with the rate adjusted to provide 99% conversion.

Br, Br, C = CH, H_3C, CH_3, COOCH, CN, O, H, H

5

investigators. Brian Jones and his group, working in the area of enzymes in organic synthesis, have used commonly available enzymes for preparation of enantiomerically pure chiral compounds. His group has used horse liver alcohol dehydrogenase (HLADH) for the production of chrysanthemic acid precursors.[11] Unfortunately, HLADH is an NAD dependent alcohol dehydrogenase. Although it catalyzes oxido reductions on a broad spectrum of substrates, the cost of NAD, at approximately $685/KG, is prohibitive since NAD is used in stoichiometric quantities. The reaction was reported on a 2 gram in which dimethyl-1,2bis(hydroxymethyl)cyclopropane (15.4mmol) was reacted with HLADH (35 mg) in the presence of 1 mmol NAD (720 milligram) and 20.3 mmol FMN. FMN in commercial grade is approximately one half the cost of NAD. Thus, although this route is an additional solution to the problem that Roussel-Uclaf has in manufacturing Decis, it is, unfortunately, still not economically possible due to the high cost of regeneration of biological energy.

Another example of the use of enzymes is the resolution of racemic mixtures which are of value as chiral synthons. This is is illustrated by the stereoselective hydrolysis of 2-acyloxy-3-chloropropyl-p-toluenesulfonate. The lipase from Psuedomonas aeruginosa was found to have a high hydrolytic activity and a stereoselectivity greater than 99% on this compound. This hydrolysis provides a route to the highly sought enantiomers of chloromethyloxirane. The novel feature involved in this stereoselective hydrolysis was the use of the toluenesulfonate as it had been previously been found that the hydrolysis of other esters gave only a 90% enantiomeric excess. This enantiomeric excess is not sufficient for the preparation of most agricultural and pharmaceutical chemicals. However, the enzymatic hydrolysis of the toluenesulfonate provides a method for certain insect hormones amd pharmaceuticals via optically active chloromethyloxiranes.

The syntheses of the compounds above are provided to illustrate to the organic chemist, that the production of chiral compounds can be accomplished in an economically feasible manner and in large quantity. Additional efforts in the use of chiral synthons for the production of agricultural chemicals is proposed as a method for reducing the toxicity and the cost of production of agricultural chemicals. In addition, these chiral synthons will open up new areas of interest to the organic chemist providing basic building blocks which may be tapped to interrupt biological mechanisms via biosynthetically important precursors.

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Chapter 16

Microbiological Identification and Characterization of an Amino Acid Biosynthetic Enzyme as the Site of Sulfonylurea Herbicide Action

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The inability to synthesize many amino acids distinguishes animal cells from those of plants, fungi and bacteria. Potential herbicidal targets include the plant enzymes responsible for synthesizing these amino acids since their selective inhibition might result in control of weed growth and low animal toxicity. Microbiological studies have recently indicated that several herbicides interfere with amino acid biosynthesis. Elucidation of the enzymic target of a sulfonylurea herbicide, sulfometuron methyl (SM), illustrates the utility of a microbial approach to studies of herbicide action. Specific reversal of SM-mediated inhibition of bacterial growth by isoleucine, methionine and pantothenate first suggested that the site of action was acetolactate synthase (ALS), a branched chain amino acid biosynthetic enzyme. Results confirming this predicition include the potent in vitro inhibition of microbial and plant ALS activity by SM, the mapping of both bacterial and yeast SM-resistant mutations to genes encoding ALS and the altered, SM-resistant forms of ALS found in microbial and plant mutants. Comparison of the DNA sequence of wild type and mutant structural genes encoding ALS from both bacteria and yeast has established the molecular bases for the herbicide-resistant phenotypes. The detailed understanding of branched chain amino acid biosynthesis in microbes has thus provided both tools and insights into the mode of SM action.

Microbes are more amenable to physiological, biochemical and genetic manipulation than metazoans and metaphytes. Diverse problems ranging from cellular differentiation and morphogenesis to sensory adaptation and multicellular development are currently being studied in microorganisms (1). A primary goal of these studies has been to develop a requisite genetic arsenal in each organism with which to

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probe these complex processes (2). Processes important to plants such as intercellular communication occurring during development and photosynthesis are being approached in microbes utilizing both classical and molecular genetics as well as biochemistry and physiology. Indeed, significant progress is being achieved (e. g. 2, 3).

Another process of importance to plant science is amino acid biosynthesis. Plants and most microbes share the capacity to synthesize the twenty common amino acids from central, key metabolites (see Figure 1). In contrast animals must ingest ten amino acids "essential" to their diet; they are unable to produce leucine, valine, isoleucine, threonine, methionine, lysine, histidine, tryptophan, tyrosine and phenylalanine. A sufficiently specific chemical inhibiting the biosynthesis of an essential amino acid thus might control weed growth and display little toxicity towards mammals. Indeed a number of herbicides interfere with the biosynthesis of the essential amino acids (4, see Table I).

Table I. Herbicides Interfering with Essential Amino Acid Biosynthesis

Herbicide	Pathway	Enzyme
Aminotriazole	Histidine	Imidazole glycerol phosphate dehydratase
Glyphosate	Aromatic	Enolpyruvylshikimate phosphate synthase
Sulfometuron methyl	Branched Chain	Acetolactate synthase
Chlorsulfuron	Branched Chain	Acetolactate synthase
Imidazolinones	Branched Chain	Acetolactate synthase

adapted from 4

Studies of amino acid biosynthesis in plants have a great advantage in comparison to work done on other important plant processes. Since amino acid metabolism has been extensively studied in enteric bacteria (*Salmonella typhimurium* and *Escherichia coli*) and a fungus (*Saccharomyces cerevisiae*) these organisms provide a convenient and powerful springboard for further investigation. The pathways were elucidated in these organisms and have been extensively analyzed (5). The requisite biochemical and genetic techniques have been developed in these organisms and technological hurdles are thus few. Tools are readily available including substrates, cofactors, structural genes, regulatory mutants, enzyme overproducing strains and protocols for enzyme purification. Detailed understanding of the stringent response (Figure 2, 6), a global regulatory circuit reorienting the bacterium's metabolism in response to a limiting supply of any amino acid (6, 7) was quite useful in elucidating the mode of sulfometuron methyl inhibition (see below). Upon amino acid limitation the *relA* product produces pppGpp and ppGpp, two nucleotides that have been called alarmones (7) since they signal a paucity of amino acids available for protein synthesis. These nucleotides stimulate expression of amino acid synthetic genes resulting in elevated intracellular levels of the enzymes

responsible for amino acid synthesis. Thus *relA* mutants are defective in this response and are thus more sensitive to a wide spectrum of amino acid antagonists than are wild type bacteria. Such *relA* mutants are therefore particularly useful for analyzing inhibitors of amino acid metabolism (7). Here we describe the identification of acetolactate synthase, a branched-chain amino acid biosynthetic enzyme (see Figure 3), as the target of the sulfonylurea herbicide, sulfometuron methyl (SM, Figure 4).

Sulfometuron Methyl

The broad-spectrum herbicide sulfometuron methyl is a member of a class of sulfonylurea analogues being developed by the Du Pont Company (8). These herbicides are characterized by extraordinarily low application rates of between 5 and 35 grams per hectare, short half lives in many soils and low toxicity towards mammals (LD50's > 0.5g/kg, 8). SM is a broad spectrum herbicide while an analogue, chlorsulfuron (CS, figure 4), selectively interferes with the growth of broad-leaf weeds while allowing wheat to proceed through its normal life cycle (8). The earliest studies done on the mode of action of these two herbicides utilized corn root tip assays (9). Incorporation of the radioactive precursors thymidine, uridine and leucine into DNA, RNA and protein was examined. DNA synthesis, but not RNA and protein synthesis, was inhibited by low levels of CS (9). Despite an extensive search, the biochemical basis for this inhibition of DNA synthesis is unknown (10).

Thus new approaches were taken. Genetic analyses of tobacco cell culture mutants resistant to SM and CS demonstrated that mutation of a single nuclear gene conferred a resistant phenotype upon tobacco but did not indicate a biochemical target. Since mutations selected in cell culture cause resistance in regenerated plants, it was suspected that CS and SM might inhibit a basic metabolic step common to all plant cells (11).

Microbiology of SM Action

The ability of SM to inhibit a variety of microorganisms on minimal and complete media was examined by a disc diffusion assay performed on solid medium (12). This simple and facile assay allows a wide range of inhibitor concentrations to be examined on a single petri dish. Both an Acinetobacter species and *Citrobacter freundii* are inhibited by SM on minimal medium; neither bacterial species is inhibited on rich media (12). The observation that SM-mediated inhibition of *C. freundii* is enhanced upon supplementation of the minimal medium by valine (12) suggested a means to achieve SM inhibition of the genetically tractable *S. typhimurium* by synergism of the herbicide with the amino acid. Indeed neither valine nor SM alone inhibit wild type *S. typhimurium*; however in combination the two chemicals severely retard the growth of this bacterium (Table II, 12).

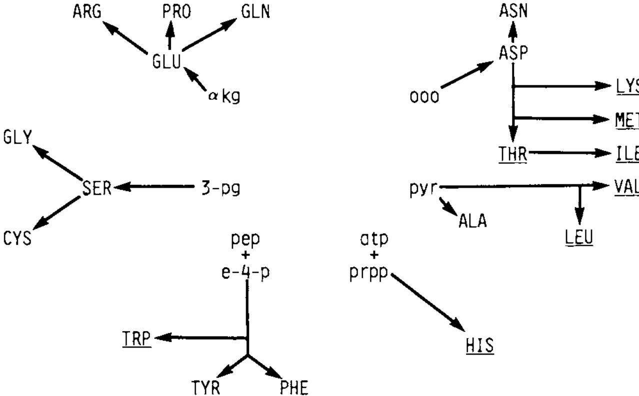

Figure 1. An overview of amino acid biosynthesis. Central metabolites are written in lower case; the amino acids essential to the mammalian diet are underlined.

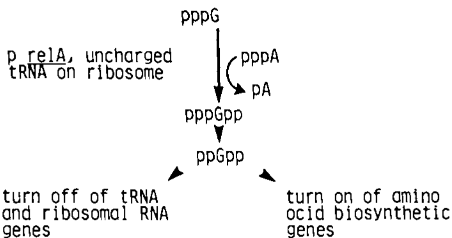

Figure 2. The stringent response.

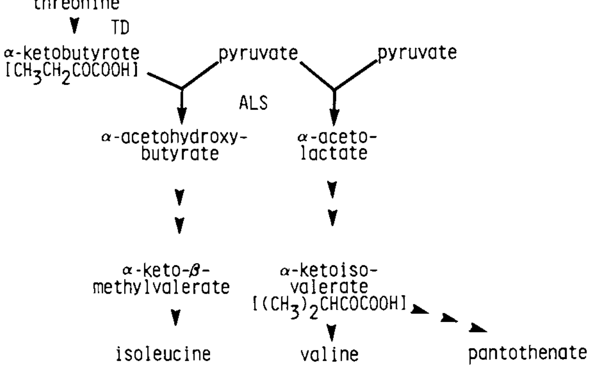

Figure 3. Branched chain amino acid and pantothenate synthesis. TD and ALS are the enzymes threonine deaminase and acetolactate synthase, respectively.

Table II. Reversal and Potentiation of Sulfometuron Methyl Growth Inhibition

Medium addition (mg/ml)	Inhibition zone[a]	
	LT2 ($relA^+$)	TA2439 (relA2)
None	<6	31
Casamino acids (0.05)	<6	<6
Isoleucine (0.083)	<6	<6
Methionine (0.05)	<6	<6
Panthothenate (0.022)	<6	9
Valine (0.083)	32	44
Valine (0.083) and Isoleucine (0.083)	<6	<6

[a] caused by 40 µg of sulfometuron methyl in a disc diffusion assay utilizing Salmonella typhimurium (12).

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The production of the alarmones ppGpp and pppGpp (Figure 5, 12) by *S. typhimurium* upon addition of valine and SM to a growing culture provides biochemical evidence for SM-mediated amino acid starvation within the cell. The inhibition of both *C. freundii* and *S. typhimurium* is prevented by inclusion of isoleucine in the growth medium suggesting that SM interferes with isoleucine production (Table II, 12). Isoleucine also prevents the SM and valine induced induction of the stringent response (Figure 5, 12). That methionine or the vitamin pantothenate also prevent inhibition of *C. freundii* and *relA* mutants of *S. typhimurium* (Table II, 12) and that auxotrophy of *S. typhimurium ilvG* mutants is satisfied by isoleucine, methionine or pantothenate (13) suggested that the target of the herbicide is an isozyme of acetolactate synthase, ALS II encoded by the *ilvGM* locus (12).

α-Ketobutyrate and the Biosynthesis of Branched Chain Amino Acids

Branched chain amino acid synthesis has been extensively studied (5, Figure 3). In branched chain amino acid biosynthesis ALS is the second enzyme of isoleucine biosynthetic pathway and the first enzyme of valine synthesis. Commonly the first enzyme of a synthetic pathway is inhibited by the end-product thus preventing waste of carbon skeletons and energy. Two ALS isozymes exist in *S. typhimurium*; ALS I is end-product inhibited by valine while ALS II is active in the presence of this allosteric inhibitor (14). If ALS II is inhibited by SM, while ALS I activity is not effected, the requirement for both valine and SM to prevent growth of *S. typhimurium* would be explained. ALS II efficiently catalyzes both the condensation of two molecules of pyruvate to form acetolactate (a precursor of valine, leucine and pantothenate) and the reaction of pyruvate with α-ketobutyrate (AKB) yielding the isoleucine precursor acetohydroxybutyrate. ALS I is, however, much more effective in forming acetolactate than in yielding acetohydroxybutyrate (13, 15, 16). Thus large quantities of the

isoleucine specific intermediate, α-ketobutyrate (AKB), accumulate upon loss of ALS II activity (13, LaRossa and Smulski, unpublished observations). AKB is produced from L-threonine in the first step of isoleucine synthesis catalyzed by threonine deaminase which is end product inhibited by isoleucine (5). AKB is toxic to cells; it has been proposed to interfere with a variety of processes including sugar transport (17), glycolytic flux (18), the TCA cycle (19), aspartate formation (19), pantothenate production through competitive inhibition of the first pantothenate-specific enzyme (Figure 3, compare the structures of AKB and α-ketoisovalerate) causing lowered capacity to synthesize methionine and lysine (13) and tRNA aminoacylation with isoleucine (20). It thus appears most detrimental for the cell to accumulate this intermediate.

Acetolactate Synthase

In *E. coli* and *S. typhimurium* the structural genes for acetolactate synthase isozymes have been isolated (21,22,23; see Table III). The 150,000 dalton bacterial isozymes (I and II) are tetrameric; within each tetramer are two large (α) and two small (β) subunits encoded by genes that lie adjacent to each other on the bacterial chromosome (21, 22). Thus the *ilvBN*, the *ilvGMEDA* and the *ilvIH* operons located at minutes 81, 83 and 3 respectively each encodes a distinct isozyme (14). The nucleotide sequences of these genes (24, 25, 26, 27) and the yeast *ILV2* gene encoding a (the only?) subunit of the single yeast isozyme (28) are known. At the amino acid level there exists substantial homology within three segments of the polypeptides from yeast and bacterial isozymes separated by two intervening segments of nonhomology (24-28, see Figure 6). The enzymes require TPP, FAD and divalent metal ion for activity; the absolute requirement for FAD in this process is not obvious since the net redox of the other reaction components is unchanged by catalysis. ALS I (22), ALS II (21) and ALS III (23) have been purified to homogeneity from bacterial strains overproducing the enzymes due to the presence of the cognate structural genes on multicopy plasmids. Gram quantities of pure *S. typhimurium* ALS II (21), obtained from an *E. coli* strain harboring the *S. typhimurium ilvGM* region on a multicopy plasmid (29), provide the needed starting material for a detailed description of the inhibitor-enzyme interaction.

Table III. Acetolactate Synthase Gene-Enzyme Relationships

Source	Isozyme	Genes	Subunit Struct.	SM[a]	Resistant Mutant
Enterobacteria	I	*ilvBN*	$(pB)_2(pN)_2$	No effect	yes[b]
	II	*ilvGM*	$(pG)_2(pM)_2$	Inhibits	yes
	III	*ilvIH*	$(pI)_x(pH)_y$	Inhibits	NT[c]
Yeast	-	*ILV2*	?	Inhibits	yes

[a] effect on enzyme activity in vitro.
[b] wild type allele.
[c] not tested; selection schemes are available.

Figure 4. The sulfonylurea herbicides sulfometuron methyl (top) and chlorosulfuron (bottom)

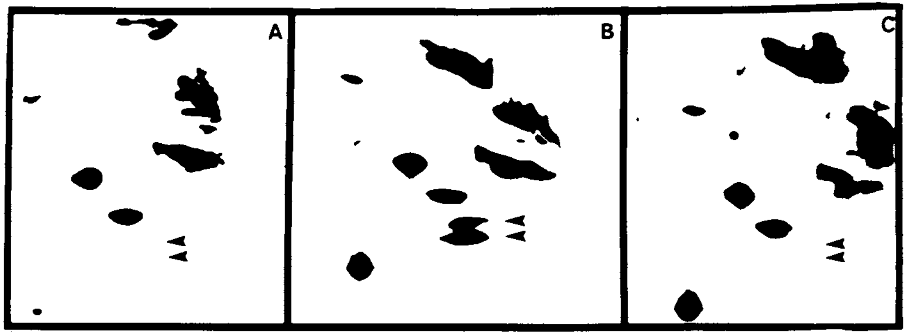

Figure 5. Sulfometuron methyl induces the stringent response. ^{32}P labeled cells of S. typhimurium growing in minimal medium supplemented with valine are untreated (panel A), treated with 100 μm SM (panel B) or treated with 100 μm SM and isoleucine (panel C). Extracted nucleotides are separated by two-dimensional TLC and visualized by autoradiography. The upper arrow in each panel indicates the position of ppGpp while the lower arrow indicates the position of pppGpp.

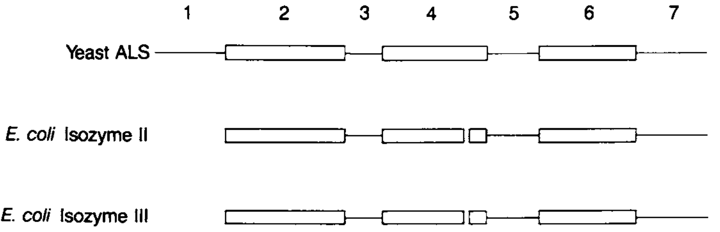

Figure 6. Amino acid sequence homology among microbial ALS isozymes. Each line represents the large polypeptide of an isozyme. Regions of homology are indicated by the bars.

Tests of ALS Inhibition by SM

Branched chain amino acids prevent SM-mediated inhibition of bacterial (12, 30), yeast (31), and pea (32) growth. This suggests that the mode of inhibition is similar in each organism. Accumulation of -ketobutyrate is observed upon treatment of *S. typhimurium* with SM and valine. Similar results have been obtained in yeast (see Figure 7). ALS activity present in each organism is inhibited by low concentrations of the herbicide (Ki's re 50nM; 12, 31-33; see Figure 8 and Table IV) although as predicted ALS I activity of *S. typhimurium* is uninhibited by 1mM SM (30, see preceding section for prediction).

Table IV. Inhibition of ALS Isozymes by CS and SM

Isozyme	K_i (CS)	K_i (SM)	References
Bacterial I	NT	$>10^6$	30
Bacterial II	NT	65	12
Yeast	NT	120	31
Pea	21	16	32
Wheat	19	NT	32
Soybean	23	NT	32
Tobacco	14	8	32,33
Green foxtail	26	NT	32
Johnson grass	36	NT	32
Morning glory	24	NT	32

K_i are reported in nM
NT: not tested.

Mutants resistant to high levels of SM have been selected in *S. typhimurium* (12), *S. cerevisiae* (31) and tobacco (11). The bacterial and yeast mutations conferring SM resistance map to the *ilvGM* (12) and *ILV2* regions (31), respectively. These genes encode *S. typhimurium* ALS II (14) and yeast ALS (31, 34). The ALS activities of these mutants and those isolated in tobacco are altered; the mutant enzymes are not inhibited by SM in vitro (12, 31, 33). In Figure 9 a comparison of the inhibition of wild type and a mutant yeast ALS is presented. Biochemical genetics thus indicates that the herbicide target is ALS in a variety of organisms. Increased gene dosage of *ILV2* in yeast (31) and *ilvGM* in *E. coli* (Yadav *et al*., unpublished observations) due to placement of these genes on multicopy plasmids results in higher intracellular ALS levels and a low level of resistance to SM. This again indicates that the primary target of SM is ALS.

Molecular Genetics of SM-ALS Interaction

To localize the yeast *ILV2* gene within the yeast insert carried on the multicopy plasmid pCP2-4-10 insertional mutagenesis by the transposon Tn5 (35, 36) was utilized. The results (Figure 10) indicate that *ILV2* maps to the rightmost 3 kilobase pairs of the yeast DNA insert in agreement with deletion (31) and mRNA (28)

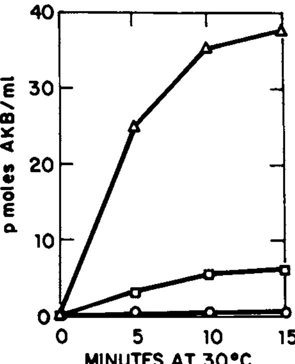

Figure 7. AKB accumulation. ^{14}C-threonine (1 μM, lmC/mMole) was added to a yeast culture growing in minimal medium. To an aliquot was added 100 μM SM (Δ) while to a second was added 100 μM SM and isoleucine, leucine and valine (40 μg/ml of each, o). The third aliquot (□) had no additions.

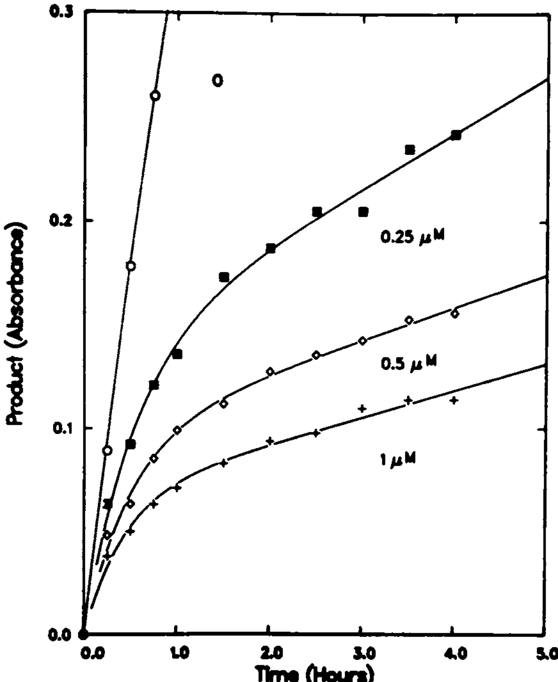

Figure 8. Inhibition of Salmonella ALS II by various concentrations of SM. (Reproduced with permission from reference 12. Copyright 1984, The American Society of Biological Chemists.)

mapping, two more laborious processes. The nucleotide sequences of an SM-resistant mutant and the wild type alleles of the yeast ILV2 and bacterial ilvGM genes have been determined. A single amino acid substitution in the first conserved region changes yeast ALS from an enzyme that is inhibited by SM to one that is completely resistant to the herbicide. Similarly each of two distinct amino acid substitutions within the first conserved segment of bacterial ALS II results in an uninhibited enzyme (37).

Isolation of Other Structural Genes for Acetolactate Synthase

As noted above, structural genes encoding ALS have been isolated from yeast and bacteria. Because of the striking homology among bacterial and fungal ALS genes (28) the yeast ILV2 gene was used as a hybridization probe to screen genomic DNA libraries prepared from diverse organisms. Putative ALS-encoding clones were isolated from the blue green alga, *Anabaena* and two higher plants, the crucifer *Arabidopsis* and tobacco (38). The cloned DNA segments obtained from the tobacco, *Arabidopsis*, and *Anabaena* libraries are shown by sequence analysis to encode ALS (38).

Why is ALS an Excellent Target for Herbicides?

Four structurally unrelated herbicides interfere with the biosynthesis of "essential" amino acids (for a review see 1). It is striking that both imidazolinone (Figure 11, 39) and sulfonylurea herbicides interfere with ALS since there are 49 discrete enzyme targets (5) involved in the biosynthesis of these amino acids. An explanation for this coincidental inhibition is that interference with ALS action leads to the accumulation of AKB. This intermediate is clearly toxic, although the precise mechanism(s) of its toxicity await elucidation. The powerful genetic arsenal available in *S. typhimurium* allows an approach to the targets of AKB. The activity of the SM-noninhibitable ALS I allows wild type *S. typhimurium* to grow in the presence of this herbicide. Isolation of 5,000 random insertion mutants of this bacterium is easy utilizing the transposon Tn*10* since the selectable phenotype of tetracycline resistance is associated with the inserting element (40). Fifteen of these insertion mutants are more sensitive to SM than the non-mutagenized parent (41). The locations of these insertions on the genetic map of *S. typhimurium* (42) has been determined by facile methods allowing rapid mapping of Tn*10* inserts (40, 43). One insertion has been shown to inactivate aspC (41), a gene which encodes the more active of two isozymes that convert oxalacetate, an intermediate of the TCA cycle, to aspartate (44). It has been hypothesized that the remaining low-velocity isozyme cannot produce enough aspartate to support growth (41) after glycolytic flux to the TCA cycle is impaired by SM-mediated accumulation of AKB (18, 19). These results provide strong genetic evidence for one proposed target of AKB, the glycolytic and TCA pathway to aspartate. Characterization of the other mutants may provide additional insight into AKB toxicity.

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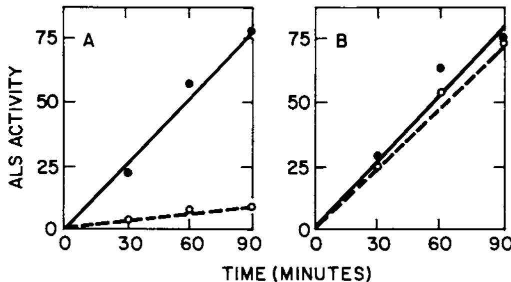

Figure 9. ALS activities of wild type (A) and herbicide-resistant (B) yeast strains. Assays were performed in the presence (o) or absence (●) of 0.8 μM SM.

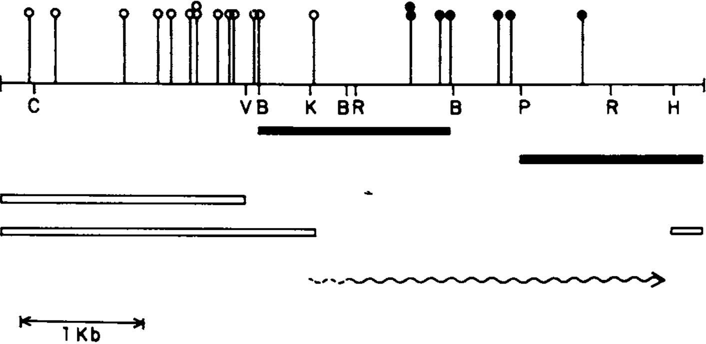

Figure 10. Map of the yeast ILV2 gene within a cloned DNA segment. Letters indicate restriction enzyme cleavage sites: C, ClaI; V, EcoRV; B, BglII; K, KpnI; R, EcoRI; P, PvuII; H, HindIII. Deletions are indicated by bars, whereas lollipops indicate the sites of Tn5 insertions. Mutations represented by filled symbols destroy ILV2 activity, whereas those depicted as open symbols do not affect ILV2 function. The extent of ILV2 mRNA is indicated by the wavy arrow.

Figure 11. An imidazolinone herbicide (from 39).

Conclusions

Microbes have played a critically important role in defining ALS as the site of SM action. The availability only in enteric bacteria of sophisticated genetic tools, such as selectable transposons for insertional mutagenesis and strain construction, presages that understanding of the role of AKB in the herbicidal activity of SM will first be determined in microbial systems. In addition, the availability of recombinant plasmids bearing microbial structural genes specifying ALS have made possible (a) the isolation of plant genes specifying this enzyme, (b) the isolation of large quantities of pure enzyme for biochemical and physical analyses and (c) determination of mutational alterations within the structural gene yielding an enzyme that is uninhibited by the herbicide.

Nonetheless unanswered questions remain. What are the relative contributions of branched chain amino acid deficiency and AKB overabundance to the cytotoxic effects of the sulfonylurea and imidazolinone herbicides? Will the delineation of the cytotoxicity of AKB towards S. typhimurium, the only system in which it has been approached, provide relevant information towards the roles of this molecule in the inhibition of plant growth? Can herbicide-resistant alleles of ALS structural genes be used as dominant selectable markers in the transformation of a wide variety of sensitive cell lines? What are the structural details of the interaction of ALS with the sulfonylurea and imidazolinone herbicides? Are eukaryotic ALS isozymes composed of nonidentical subunits? How does SM cause cessation of DNA synthesis in plants? In the next few years answers to some of these questions may emerge.

The use of microbes to discover targets of herbicide action is not limited to the sulfonylurea herbicides. Microbial experiments also have made important contributions to the discovery of the sites of action of both glyphosate (45-48) and aminotriazole (49). With the development of sophisticated prokaryotic molecular genetics in numerous organisms that perform a variety of diverse functions, more and more processes will be dissected by microbial molecular biology.

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Chapter 17

Genetic Engineering of Bacterial Insecticides

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Bacterial insecticides, especially *Bacillus thuringiensis*, have become important factors in insect control programs because of their efficacy and safety. *B. thuringiensis* produces a toxic protein that assembles into a crystal. Depending on the *B. thuringiensis* strain, the toxin is specific for lepidoptera, mosquitoes, or beetles. Genes for the toxin have been cloned from several *B. thuringiensis* strains, analysed in detail, and manipulated by recombinant DNA techniques. The toxic regions of these crystal proteins have been identified and sequenced. Efforts to improve the insecticidal properties of the toxin protein concentrate on yield improvement, expression in alternate hosts such as plants, and protein sequence changes. Progress toward these goals has been good, but more knowledge of the insect-toxin interactions is required for a major breakthrough.

Biological pesticides are becoming recognized as an important factor in crop and forest protection and in insect vector control. These pesticides are natural, disease-causing microorganisms such as viruses, bacteria and fungi, that infect or intoxicate specific pest groups. Biological pesticides have great biological diversity, but nonetheless share several characteristics. First, they affect a narrow spectrum of pests, usually within a single order or even family. Second, they are very safe, due in large part to their narrow spectrum of activity. They do not cause diseases in vertebrates, and usually have no effect on predator species. Third, they share certain limitations. Most are slow acting compared to chemicals, and since many must be infective in order to control pests, formulations must provide long term

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viability. Also, the insecticidal microorganisms or their toxic products are sensitive to environmental factors such as ultraviolet light, plant surface chemicals, heat, and dessication.

The greatest successes in microbial pesticides have come from uses of the insecticidal bacteria, Bacillus thuringiensis strain HD-1 subspecies kurstaki (or BTk), and B. thuringiensis serotype H-14 subspecies israelensis (or BTi). BTk is effective against foliage feeding caterpillars of which over 150 have been documented, with the most notable being the cabbage looper, tobacco hornworm, tobacco budworm, European corn borer, gypsy moth, and spruce budworm. BTi on the other hand is relatively non-toxic to lepidoptera, but is very effective against mosquitoes and blackflies. In both cases B. thuringiensis acts as a stomach poison with the combined action of a protein toxin and septicemia due to germination of spores and bacterial proliferation within the insect. The toxicity, production, and use of B. thuringiensis has been succinctly reviewed by Krieg and Miltenburger (1). Uses for which BTk is an accepted insecticide range widely and include forestry, vegetables, corn, tobacco, ornamentals, fruit trees, and stored grains. Sales of BTk are in the range of $15 to $25 million annually. The success of BTk is based on a combination of efficacy and safety. For pests such as cabbage looper, gypsy moth, and tobacco budworm, excellent control can be acheived with less than 10 grams of BTk spores and crystals per acre. Control of European cornborer with a granular formulation meets or exceeds the performance of the chemical insecticide alternatives.

The safety of BTk is not only beneficial environmentally, but also has practical consequences. Unlike most other insecticides, BTk does not require special protective clothing, there is no waiting period before re-entering the field, and it may be applied up to the day of harvest. BTk is non-toxic to bees, and, because it does not harm predatory insects, is ideally suited to integrated pest management programs. Furthermore, it can be used for aerial spraying of residential areas for control of gypsy moth, without fear of harm to humans or pets.

The acceptance of BTk as an insecticide is best illustrated by its use in gypsy moth control programs in the U.S. When introduced in 1981, BT was applied to 22,000 acres of gypsy moth infected forest, about 6% of treated acres. In 1985 and 1986 over 900,000 acres were treated annually, accounting for more than 70% of all pesticides applied during that time. Because of the intense environmental concern surrounding gypsy moth programs, and the demonstrated efficacy of BTk formulations, it is anticipated that BTk will further displace chemical insecticides in forestry.

BTi is also gaining inceased acceptance as a larvicide for mosquitoes and blackflies. Like BTk, BTi is highly specific. While controlling most mosquito and blackfly species, it does not have appreciable toxicity to most other flies, and under field conditions is harmless to non-dipteran insects. This environmental safety, coupled with a lack of human toxicity, makes BTi an ideal control agent for these biting pests. Blackflies

infest fast moving streams which can be important as drinking water or for sport fishing. Therefore, chemicals with any suspected mammalian toxicity or carcinogenicity, or that could wipe out insect populations necessary as diet for game fish, would be unacceptible. BTi is now being used in large programs to control blackflies in the U.S. and Canada, and under World Health Organization auspices in West Africa.

The combination of safety and efficacy also make BTi an attractive material for use in mosquito control. Most mosquito insecticides are used only near residential areas, necessitating the use of harmless materials. In addition to being safe to humans, BTi does not harm beneficial insects or crustaceans often found in mosquito habitats. Because BTi is so effective on its targets (use rates are as low as 5g/Acre) it is anticipated that use of BTi will soon be the predominant insecticide for mosquito larva control.

Other bacterial insecticides exist, but have not yet acheived the commercial success of BT and BTi. *Bacillus sphaericus* produces a protein that is toxic primarily to Culex and Anopheles mosquitoes, and has the potential for long term residual control. Formulations of *B. sphaericus* are under development and are expected to be available for public health use in 1987. *Bacillus popilliae* and *Bacillus lentimorbus* both infect the larvae of scarabaeid beetles, including Japanese beetle grubs, causing milky disease. Both of these species are registered with EPA as biorational insecticides. Unlike *B. thuringiensis*, both species are obligate parasites and can be raised only in beetle larvae. Until a means of host-independent growth and sporulation of these species is developed, it is unlikely that they will be used on a broad scale. The use of *B. popillae* and *B. lentimorbus* for grub control is reviewed by Bulla *et al.* (2).

The Molecular Biology of Bacterial Insecticides

The insecticidal toxins of *B. thuringiensis* and *B. sphaericus* are proteins, referred to as δ-endotoxins. These proteins form crystalline structures, such as that shown in figure 1, during sporulation. Despite having the common features of crystal formation and selective insect toxicity, the δ-endotoxins show considerable diversity between different strains. The lepidopteran specific strains have endotoxin proteins in the range of 130,000d to 140,000d [for review see(3)]. For instance, B.t. HD-73 has a δ-endotoxin with molecular weight of 133,000 (4); *B.t. sotto* has a δ-endotoxin with molecular weight of 144,000, (5) and B.t. HD-1 has two δ-endotoxins with molecular weights 130,000 and 135,000 which are the products of separate genes (6, 7).

The 130-140,000d δ-endotoxin is not toxic in its native form, but must be cleaved to a 60-65,000 fragment to be active. This cleavage takes place in the insect gut. After ingestion by a caterpillar, the proteinaceous crystal dissolves in the alkaline gut juices. Digestion by gastric proteases then cleave the

protoxin into the 65,000d active form (8). The combined requirements for an alkaline environment for crystal dissolution and appropriate proteases for δ-endotoxin activation contribute to the varying degree of toxicity of BT to various lepidoptera.

The δ-endotoxin of B.t. israelensis (H-14) is not as clearly understood as that of BTk. The parasporal crystal of BTi contains several proteins with molecular weights ranging from 26,000 to over 100,000d (9). Several authors have reported that the 26,000d protein has insecticidal activity (10, 11, 12). However, other reports suggest that the toxin is 66,000d, (13, 14). Because BTi has hemolytic and cytolytic activities in addition to insecticidal activities (15) the possibility exists that multiple toxin proteins exist in the crystal. However, evidence from genetics (12) and analysis of isolated proteins (16) tends to rule out a major role of the 65,000d protein as the primary insecticidal toxin. Unlike the lepidopteran-specific BTk toxin, the 28,000d BTi protein does not appear to be derived from a higher molecular weight polypeptide (17).

A third class of B. thuringiensis termed Pathotype C has recently been discovered. (18, 19). This pathotype, now represented by 2 subspecies, B.t. tenebrionis and B.t. san diego, has a crystalline protein that is toxic to beetles, but not to mosquitoes or lepidoptera. The crystal in pathotype C has a rectangular structure and is composed almost entirely of a 64,000d protein (19). No higher molecular weight precursors to the 64,000d protein were detected.

The insecticidal proteins of the three major B.t. pathotypes are all different. None of the three cross-react immunologically with the others (20, 19). A sequence of 30 amino acids of the BTi toxin protein has no relationship the the lepidopteran specific BTk toxin sequence derived from the nucleotide sequence of the gene (10). However, a gene that encodes a toxin protein from Bti has been sequenced, and the derived protein has substantial homology with a short region of the δ-endotoxin protein from B.t. HD-1 (21). However, it shares no homology with the 26,000d protein from BTi. Indeed, it is not clear which of the BTi crystal proteins it might correspond to, since this gene encodes a 58,000d protein, which has not been reported as a constituent of the BTi crystal.

B. sphaericus is another species that produces a proteinaceous crystal which is toxic to mosquitoes (22). In B. sphaericus, the crystal is enclosed within the exosporium and so is directly associated with the spore, unlike most B. thuringiensis subspecies where the crystal and spore are separate. The B. sphaericus crystal is made up of several proteins with molecular weights ranging from 43,000d to well over 100,000d (23). Only proteins of 43,000d and 63,000d remained after solubilization at high pH, and of these two, only the 43,000d protein showed insecticidal activity. It is probable that the 43,000d toxin protein was derived from a 110,000d precursor and that, in the insect gut, the 43,000d protein is cleaved to a 40,000d product.

Recent evidence indicates that cytolytic activity of the B. sphaericus toxin requires pre-treatment with mosquito gastric juices (24), suggesting that proteolysis is required for toxin activation, as is the case with the lepidopteran specific BTs. The B. sphaericus toxin protein has no homology with BT toxins by the criteria of immunoreactivity (23) or DNA hybridization (25). However, data from DNA or protein sequence comparisons will be necessary for an examination of possible distant relationships.

Genetic Engineering of Bacterial Insecticides

The δ-endotoxin gene from BTk was first cloned by recombinant DNA techniques in 1981 (26). Since then, the toxin genes have been cloned from a variety of B.t. strains and from B. sphaericus. A list of these cloned genes is in Table I. The isolation of these genes enables both the analysis and manipulation necessary to alter or improve the insecticidal proteins.

A major target in toxin gene analysis has been to identify protein regions that provide the toxicity or insect specificity of the δ-endotoxins. To date the complete DNA sequence of toxin genes from 4 B.t. strains have been reported (Table I). The structure of cloned genes and their regulatory elements have been well described in recent papers (27, 28).

When the B.t. δ-endotoxin is proteolytically activated in the insect gut, it is cleaved to a primary product of approximately 60,000d. By analysis of proteins synthesized from experimentally truncated, cloned genes, it has been shown that the active portion of the δ-endotoxin is at the amino end of the protein (29, 4, 30, 31). Schnepf and Whiteley working with BTk found that removal of the first 50 amino acids of the δ-endotoxin protein eliminated toxicity, but that removal of only the first 10 did not. At the carboxy-terminal end, truncation of the protein at amino acid 603 eliminated toxicity whereas truncation at amino acid 645 did not (29). Thus, the toxic moiety is between amino acids 10 and 645. Others working with a gene from B.t. HD-73 were able to truncate to amino acid 612 without losing toxicity (4). Because the genes analysed by the two groups differed from each other in nucleotide sequences, the results are not directly comparable.

A comparison of the reported B.t. toxin genes indicates that sequences of genes have diverged from each other in some regions more than in other (28). Interestingly, these regions can be correlated with the toxic and non-toxic parts of the protein. The first third of each of the proteins (from the amino terminus) share virtually identical sequences. However, the center third shows considerable divergence from sequence to sequence with some regions of nearly complete non-homology. The carboxy-terminal third of the genes re-establish homology, although not as tightly as the amino terminus. The point at which the non-homology of the central sequence changes to the homology of the carboxy-terminal third is at amino acids 600-610, coincident with the terminus of the toxic moeity. The evolutionary conservation of the

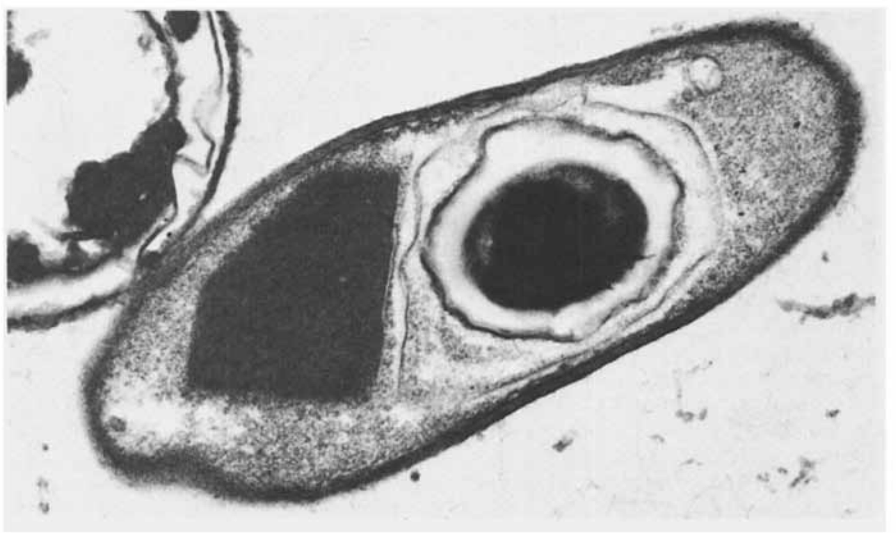

Fig. 1. Electron micrograph of B. thuringiensis HD-1 showing crystal and spore.

Table I. Cloned Genes for Bacterial Insecticidal Proteins

Serotype	Sequence Available	Reference
B.t. kurstaki HD 1	Yes	7, 21, 26, 34, 35
B.t. kurstaki HD 73	Yes	4, 6
B.t. berliner 1715	No	32
B.t. sotto	Yes	5, 36
B.t. aizawai	No	36
B.t. subtoxicus	No	36
B.t. san diego	No	19
B.t. thuringiensis HD 2	No	6
B.t. israelensis IPS 78	No	17
B.t. israelensis ONR60A	Yes	21
B.t. israelensis 1PS 82	Yes	11
B.t. israelensis HD 567-1	No	38
B. sphaericus	No	39

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carboxy-terminal region of the δ-endotoxin suggests that it must have biological function, but that function is not directly related to toxicity and is unknown.

The information on the structure and toxicity of the δ-endotoxin genes has been useful in designing experiments to alter the activity or function of these proteins. In general, the genetic engineering approaches to bacterial insecticides fall into three categories. The first is to change the yield or potency of existing bacterial toxins. Second is to change the host organism that carries and expresses the genes. And third is to change the spectrum of toxicity, to alter or increase the insecticidal range. Because of the amount of information available on lepidoptera-specific BT δ-endotoxins, almost all of the published genetic engineering work has been on these proteins. Although the current efforts on BT genetic engineering are intense, the number of research reports is small, primarily because most work is now being done by commercially oriented organizations rather than universities.

The δ-endotoxin of BT accounts for over 30% of the protein of the sporangium. Therefore, efforts to increase the overall synthesis are unlikely to result in substantial yeild improvement. Nevertheless, current studies to elucidate the regulatory elements of δ-endotoxin genes may lead to useful production increases. The promoter regions from several B.t. genes have been analysed and all appear to have identical sequences (4, 5, 27). Wong, Schnepf and Whiteley found that two promoters function in *B. thuringiensis*, one early in the stationary phase and one in mid and late stationary phase. A third promoter is active fortuitively in *E. coli*. In *B. subtilis*, cloned δ-endotoxin genes can be expressed at high level (7) but the location of the promoter has not been reported. In fact, the fortuitous *E. coli* promoter has more homology to a consensus *B. subtilis* promoter than do either of the *B. thuringiensis* promoters. The organization of promoter sequences in Bt have been reviewed recently (28). Despite the work on the structure of the δ-endotoxin gene promoters, no altered genes with enhanced δ-endotoxin synthesis have been described.

An alternative approach to increasing δ-endotoxin yield is to reduce the time required for a B.t. fermentation cycle. Recently we demonstrated that a δ-endotoxin gene from BTk can direct the synthesis of crystals in *B. subtilis* without a requirement for stationary phase of growth (Figure 2) (7). This contrasts with results from a cloned gene from B.t. *berliner* in which the δ-endotoxin was synthesized only during sporulation (32). The cloned B.t. *berliner* gene is flanked by several thousand base pairs of B.t. DNA, including a region of substantial secondary structure near the promoter sequence (33). The BTk gene, on the other hand was constructed with very little B.t. DNA adjacent to the promoter (7) suggesting that upstream sequences may be involved in sporulation-restricted expression. Expression of B.t. genes during vegetative growth might shorten the overall time

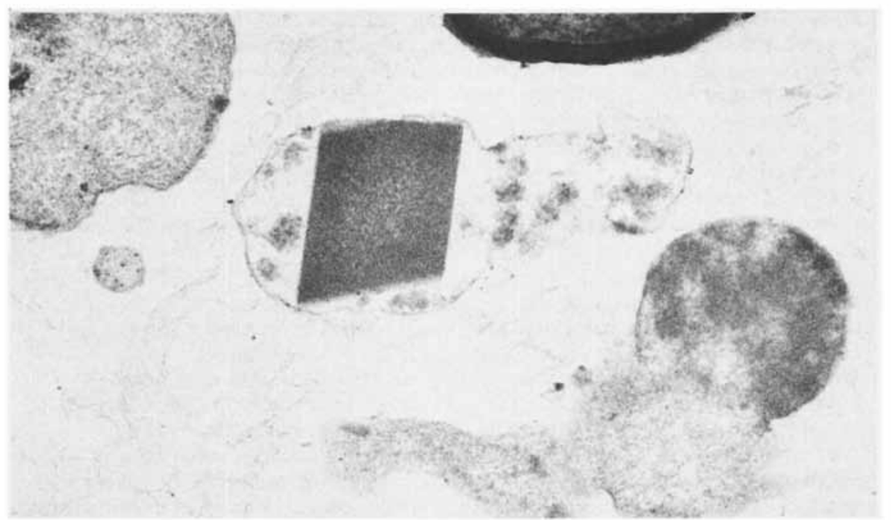

Fig. 2. Electron micrograph of B. subtilis which carry a B. thuringiensis HD-1 gene on a recombinant plasmid. A protein crystal is visible.

required for maximum crystal production, or allow for continuous fermentation processes.

Recently several groups have been successful in transferring B.t. δ-endotoxin genes into alternate host organisms to substantially change the means of application of the insecticide. In a much publicized project, Monsanto scientists have introduced a cloned B.t. δ-endotoxin gene into the corn root colonizing bacterium, Pseudomonas fluorescens (34). This, in concept, can be used to deliver the insecticidal crystals beneath the surface of the soil in areas where root feeding insects do the most damage. Unfortunately, because of restrictions to the use of such recombinant organisms in the environment, the utility of this approach has not yet been demonstrated.

A second use of Ps. fluorescens has been developed by a research group at Mycogen Corp. Ps. fluorescens will express the B.t. δ-endotoxin at high levels when it carries the B.t. gene on a recombinant plasmid. Unlike B. thuringiensis however, Pseudomonas does not sporulate. The recombinant Pseudomonas can be killed during production, thus providing a source of recombinant but non-viable bacterial insecticide. Because of the non-viability of the product, the Environmental Protection Agency has approved field tests for this material. Mycogen scientists believe that encapsulation of the B.t. crystal by Pseudomonas cell wall will protect the δ-endotoxin from environmental factors. Performance data on this material in the field are not yet available.

An exciting new approach to the use of B.t. δ-endotoxin has been the introduction of B.t. genes into the genome of a plant. Groups at Agrigenetics Corp and Plant Genetic Systems have transferred B.t. δ-endotoxin genes into a plasmid of the infectious bacterium Agrobacterium tumifaciens and used the Agrobacterium to introduce this plasmid into tobacco plants. The results indicate that not only do plant tissues synthesize δ-endotoxin, but the leaves are also toxic to the tobacco hornworm. As methods for introducing foreign genes into plants become available for more species, this could become a widespread method for crop protection.

Improvement of bacterial insecticides through protein engineering to alter the spectrum of insects that can be controlle is the major long term goal of many reseach programs. It is not a easy task. As yet, no results have appeared in either the scientific or popular literature. Some short-term approaches have been suggested, such as changing the size of the δ-endotoxin through gene truncation, or forming chimeras of genes from different subspecies to make combinations not found in nature. As a model system, it was recently shown that a gene made by fusion of part of the δ-endotoxin gene and part of a drug resistance gene will express both activities (30).

The more long range approach involves directed mutagenesis of δ-endotoxin genes. This will result in replacement of amino acid residues or additions or deletions to the toxic protein. The technology for mutagenesis at specific sites within a gene is well

developed, and the ability to alter protein function through such procedures has been demonstrated. However, site-directed mutagenesis of δ-endotoxin genes to make new insecticides is a particularly difficult task. The limited knowledge of the protein structure in insect toxicity makes the exact identification of appropriate sites for mutation impossible. As an alternative, random mutations can be made within the 600 amino acid region known to be toxic. The problem here is the high cost and relative imprecision of the assays needed to screen the mutant proteins for altered insecticidal activity.

The greatest limitation to advances in genetic engineering of bacterial insecticides is knowledge. The techniques for gene isolation, analysis and manipulation are well developed and in wide use. However, we are generally ignorant of the biological properties of the bacterial toxin proteins and their interactions with insects. The nature of target sites within the insect gut is unknown. Data on the mode of action of δ-endotoxins is diverse and conflicting. Certain lepidopterans are more susceptible to some strains of B.t. than to others, but the biochemical or molecular basis of this specificity is unknown. Bacterial insecticides are an attractive alternative to conventional pesticides and are an excellent target for improvement by genetic engineering. With integrated efforts in molecular biology and insect physiology we should see significant developments in bacterial insecticides in the next five or ten years.

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Chapter 18

Ice Nucleation-Deficient Bacteria as Frost Protection Agents

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Deletion mutants of *Pseudomonas syringae* and *Pseudomonas fluorescens* were developed for biological control of related, ice-nucleating strains. The deletions, constructed *in vitro* and introduced into the bacteria by marker exchange, ensured that the controlling strains were deficient in ice nucleation. In other diagnostic and colonization tests the mutant strains were indistinguishable from their wild-type progenitors. These data form a basis for assessing the potential benefits of using the constructed strains as frost protection agents, and for considering the issue of safety in releasing the recombinant bacteria into the environment.

In liquid water between 0°C and -40°C, ice formation requires a heterogeneous nucleation event: molecules other than H_2O must participate in forming an initial ice template. Ice crystals of the small sizes likely to occur spontaneously above -40°C are inadequate as templates, because the growth of an ice crystal is thermodynamically unfavorable when it possesses a sufficiently large surface-to volume ratio (1). Thus an ice nucleus is something which promotes the formation of a crystal large enough that crystal growth, rather than dissolution, becomes thermodynamically favored. At relatively warm temperatures (above -6°C), it is believed that this can only be achieved by providing a template with the capability of quite accurately lattice-matching with a large number of water molecules in an ice-like configuration.

Ice nucleation is important to agricultural biotechnology because ice-nucleating bacteria increase the probability of frost damage to plants (2,3), and because it may be possible to alter that situation through bacterial engineering. Frost damage is usually caused by the mechanical disruption resulting from ice crystallization, rather than by low temperatures *per se*. The tissues of most plants can supercool to between -6 and -8°C, and thereby avoid such freezing injury, unless freezing is initiated

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exogenously. Unfortunately, exogenous ice nuclei are provided in abundance by bacteria of epiphytic growth habit: *Pseudomonas syringae*, *P. fluorescens*, and *Erwinia herbicola* (3,4,5). Most field conditions in temperate climates are conducive to colonization by such epiphytes, which will then short-circuit a plant's latent capacity to survive low temperatures by freeze-avoidance. The elimination of such bacteria would clearly provide a large degree of frost protection - but complete elimination is an unrealistic goal. However, thanks to the spectrum of ice nucleation activity (INA) shown even by clonal populations (Figure 1), the reduction of ice nucleator populations will provide a smaller, but still significant, degree of frost protection.

How can the populations of these bacteria be reduced? Various bactericides and ice nucleation-inhibiting compounds may be effective at reducing frost injury to plants (6,7,8). Biological control is also an attractive option since biological agents can propagate and therefore they may require fewer applications than chemicals; they are also less likely to be phytotoxic or to contribute to environmental pollution. Bacteriophages can reduce the INA titre *in vitro* (9), and they are worth testing in a field situation; but it must be noted that although bacteriophages have been known much longer than antibiotics, there are few if any examples of their effective use in practical situations (10). A third approach, that of using competitive exclusion to reduce the populations of ice nucleators, is the subject of this contribution.

Construction of Ice Nucleation-Deficient Competitor Bacteria

Why use recombinant DNA to construct the competitors? INA$^-$ epiphytic colonizers occur naturally, and can also be derived from INA$^+$ strains by chemical mutagenesis. Strains of both origins have been demonstrated to be capable of providing some degree of frost protection (7,11). Why then is it necessary to construct competitors by genetic engineering? One rather cynical misconception is that genetic engineering can improve only the patentability of the microorganisms.

It seems unlikely that naturally-occurring ina$^-$ bacteria would occupy the exact same niches as INA$^+$ strains, even though they may be epiphytic colonizers of the same plant species. (This argument is based on the supposition that evolutionary maintenance of the ice nucleation gene has required selection for the ice nucleation phenotype. There is little direct evidence for this idea, and one goal of our research is to test it. Another way to examine this view would be by determining whether most naturally occurring INA$^-$ and INA$^+$ bacteria constitute genetically distinct populations.) Since niche exclusion is probably essential for effective competition, a naturally-occurring INA$^-$ bacterium is therefore likely to be inferior to one that is isogenic with an INA$^+$ strain, except at the locus controlling INA. Chemical mutagenesis is not a satisfactory way to obtain such isogenic strains. For practical reasons it is common to use chemical mutagens at concentrations which are likely to cause multiple mutations; the additional mutations which do not affect INA are likely to be undetectable in the laboratory, but affect fitness adversely in the field. The

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possibility of unknown mutations being present will always complicate experiments with chemically-induced mutants. Genetic engineering offers the means to obtain truly isogenic INA$^-$ mutants and thus perform more straightforward experiments, with increased chances of success. In addition, we can engineer deletions, which will be non-revertable; this is considered a desirable trait in an organism which is scheduled for deliberate release into the environment.

Cloning of the INA genes from Pseudomonas syringae and Pseudomonas fluorescens. A gene conferring ice nucleation activity was first cloned from *P. syringae* strain Cit7 (12). We identified the corresponding genes, by DNA hybridization with the cloned Cit7 gene of plasmid pICE1.1, in naturally-occurring isolates of *P. syringae* (S203) and *P. fluorescens* (MS1650). In each case the hybridizing sequence was shown to be present as a single copy per genome. The INA genes were cloned from these strains by size-selection of restriction-endonuclease-digested genomic DNA, followed by cloning into *E. coli* plasmids and screening for ice nucleation activity. (The INA gene from S203 was obtained on an 8.5 kb EcoR1 genomic fragment, cloned into EcoR1-linearized vector pUC8 (13). The INA gene from MS1650 was subloned on a 9.0 kb EcoR1-Sal1 genomic fragment, present in cosmid pJJINA (14).) The INA genes isolated from the different strains each possessed a distinct distribution of restriction endonuclease sites (Figure 2).

Making deletions within the INA regions in suitable mobilization vectors. Our method for marker exchange in *Pseudomonas* requires the conjugal transmission of the introduced marker from *E. coli* in a nonreplicating plasmid. The requirement for transmissibility made necessary the recloning of the ice genes from the original plasmids into different vectors. Then the dissimilar restriction maps of the two genes required distinct methods for introducing an Ice$^-$ deletion into each. These steps are described below.

(a): Gene from S203. The gene was recloned into the EcoR1 site of vector pBR325 (15) on the same 8.5 kb EcoR1 fragment. Two Sal1 fragments were deleted (coordinates 1.3 to 2.4 in Figure 2) by partial digestion with Sal1, followed by religation. This removes 1.1 kb from the 3.6 kb gene which we know to be essential for ice nucleation activity in S203 (16). The final deletion construction is called pRLG13.

(b): Gene from MS1650. The gene was recloned in the same 9.0 kb EcoR1-Sal1 fragment into vector pLVC18 (17). A deletion was introduced by Hpa1/Bal31 digestion, followed by religation. This resulted in the loss of the region between coordinates 6.0 and 7.5 in Figure 2. Thus it removes 1.5 kb from the 4.0 kb gene which we have mapped in the region from MS1650 (14). The final deletion construct is called pLVC40.

Plasmid mobilization and marker exchange. The respective deletion-containing sequence was substituted for the wild-type gene in each *Pseudomonas* strain by double reciprocal recombination to the unaltered flanking regions immediately adjacent to the deleted sequence. In this way we avoided the possibility of creating any

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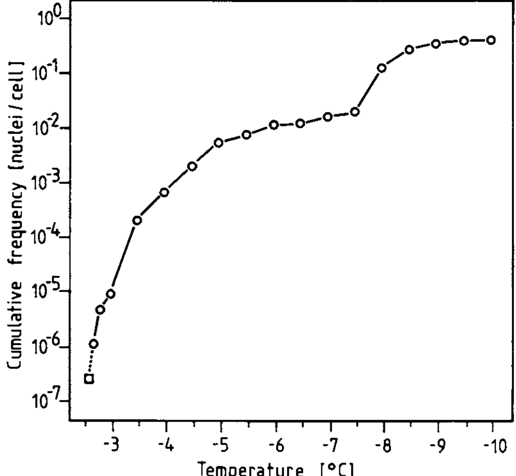

Figure 1. Spectrum of ice nucleation frequency displayed by a genetically homogeneous population of bacteria: E. coli strain JC10291 with plasmid pRLG12 (16). The source of the inaZ gene in pRLG12 is P. syringae S203, which gives an almost identical spectrum: therefore the phenotypic heterogeneity is unlikely to be due to genotypic heterogeneity. The graph indicates that very few nuclei are active at the warmer temperatures; nucleation frequency increases by orders of magnitude towards cooler temperatures. Therefore, in small bacterial populations such as occur on leaves, the probability of nucleation at a given temperature will be reduced when the mean population size is reduced. Frost protection may be assessed by the temperature difference between the "warmest" nuclei present with and without the use of the protection agent.

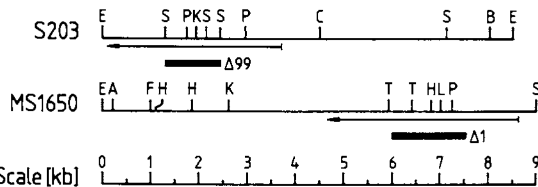

Figure 2. Restriction maps of the regions cloned from P. syringae and P. fluorescens. Solid bars indicate the region deleted for the marker exchange experiments. A: BalI; B: BamHI; C: BglII; E: EcoRI; F: EcoRV; H: HpaI; K: KpnI; L: SphI; P: PvuII; S: SalI; T: ScaI. An arrow indicates the region essential for ice nucleation in each case, and its direction of transcription.

new sequence arrangements by the recombination of heterologous DNA. Our method achieved the double recombination in a two-step process, as detailed below. The principle of the process is illustrated in Figure 3. Figure 4 shows a Southern blot analysis of the *Pseudomonas* strains before manipulation, then after stage *a*, and finally after stage *b*. This analysis provides confirmation that the marker exchange occurs as described.

(Stage *a*): Integrative recombination. pRLG13 and pLVC40 were mobilized into S203 and MS1650 respectively, with selection for tetracycline resistance. (The R64drd11/pGJ28 system (18) was used for pRLG13. Plasmid R751 (19) was used to mobilize pLVC40.) Tc^r transconjugants were obtained at a frequency of approximately 10^{-8} per recipient cell. Since the transferred plasmids could not replicate in *Pseudomonas*, these transconjugants arose from the comparatively rare events of plasmid integration into the chromosome. Two kinds of evidence indicate that the plasmid::chromosome cointegrates occur by single events of homologous recombination: firstly, no Tc^r transconjugants were obtained when a control was performed in which the transferred plasmid lacked homology, and secondly, the Southern blot analyses in Figure 4 are most readily explained by single crossovers.

(Stage *b*): Excisive recombination. The cointegrate strains were grown without antibiotic selection for 30 generations in L-broth, and then plated at a density of 400 CFU per plate. After growth to small colonies, these were replica-plated onto L-agar plus tetracycline. Comparison of the masters with the replica plates revealed tetracycline-sensitive colonies at a frequency of approximately 0.1%. From the S203::pRLG13 strain, approximately 5% of the Tc^s colonies were phenotypically Ice^-. For the MS1650::pLVC40 strain, the frequency was 20%. All the Tc^s clones result from a second event of homologous recombination, which excises the region of DNA containing vector sequences and permits its loss. However, only those crossovers which occur on the opposite side of the deletion, relative to the position of the integrative crossover, can leave the deletion behind as a chromosomal allele. A majority of second crossovers are expected to occur in the same interval as the first: hence the comparatively low ratio of Ice^-:Tc^s. Southern blots showed that the Ice^-, Tc^s strains had the expected chromosomal structure, and retained no homology to the temporarily inserted vector sequences.

Characterization of the INA-Deficient Competitor Bacteria

Testing in vitro. The INA^- strains were extensively characterized in order to verify that they were identical to their INA^+ progenitors in every trait except ice nucleation activity. Eighteen standard taxonomically significant tests were performed as described previously (20,21,22). The strains were also screened for utilization of 31 substrates as sole sources of carbon, production of bacteriocins and the phytotoxin syringomycin and sensitivity to 19 antibiotics. Each INA^- strain was identical to the INA^+ strain from which it was derived in all traits except ice nucleation activity (Table I). Growth rates of the parental strains and their derivatives in broth media were indistinguishable.

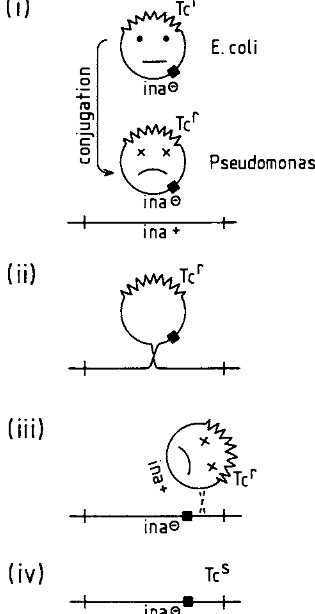

Figure 3. Strategy for marker exchange. Zig-zag lines denote the vector portion of the suicide plasmid. (i) A narrow-host range (suicide) plasmid, carrying a deletion allele of the <u>ina</u> gene, is transferred from <u>E. coli</u> to <u>Pseudomonas</u> (where it cannot replicate). (ii) Selection for Tc^r selects cells where the Tc^r marker has been rescued by a single crossover between plasmid and chromosome. Stage "a" is now complete. (iii) Growth without antibiotic selection permits the appearance of cells in which the plasmid was excised by a second, single crossover, and (iv) was subsequently lost. There are two possible outcomes: either the deletion allele substitutes the wild type allele, or the wild type allele is restored. Stage "b" is now complete.

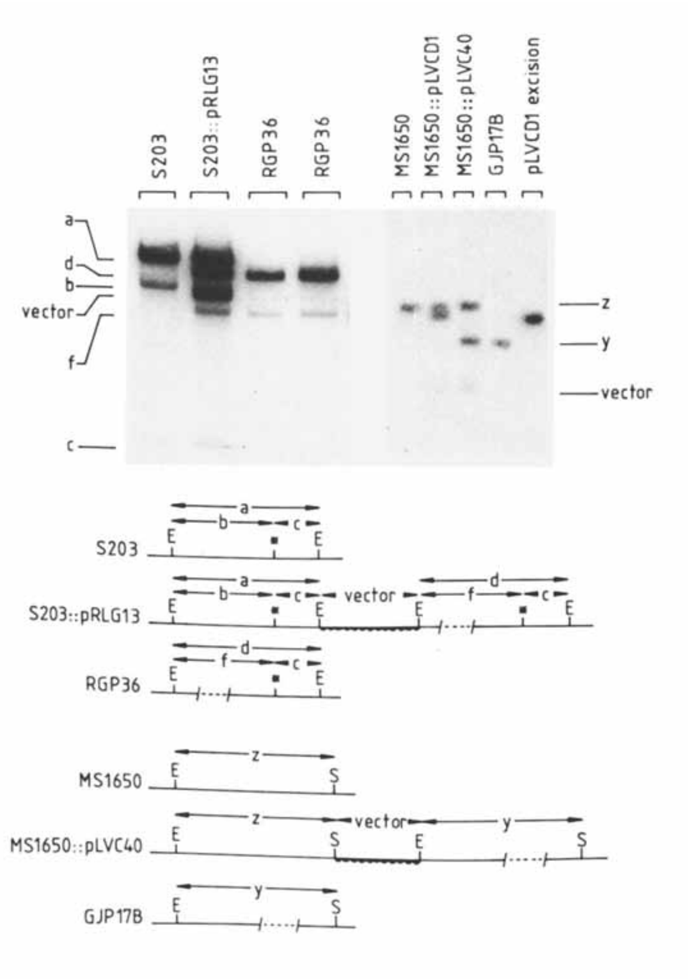

Figure 4. Evidence for the chromosomal rearrangements. Above: Southern blots probed with pRLG13 (left) and pLVC40 (right). Below: relationship of observed fragments to predicted structure at each stage. Lower case letters label corresponding fragments above and below. E: EcoRI site. S: SalI site. *: strong EcoRI* site (note that only about 10% of molecules in a population are cut at this particular site). pLVCD1 carries a deletion allele analogous to that in pLVC40; it is shown here for comparison only.

Table I. Reaction of Wild-type INA+ and INA- Deletion Mutant Strains of *Pseudomonas syringae* and *P. fluorescens* Biovar I in Diagnostic Tests

Characteristic	*P. syringae*		*P. fluorescens*	
	S203	RGP36	MS1650	GJP17B
Gram reaction	-	-	-	-
Cell shape	rod	rod	rod	rod
Glucose fermentation	-	-	-	-
Fluorescent pigments	+	+	+	+
Pyocyanine	-	-	-	-
Growth at 41°C	-	-	-	-
No. of flagella	>1	>1	>1	>1
Oxidase reaction	-	-	+	+
Arginine dihydrolase	-	-	+	+
Potato rot	-	-	+	+
Levan from sucrose	-	-	+	+
Tobacco HR	+	+	-	-
Denitrification	-	-	-	-
Gelatin hydrolysis	-	-	+	+
Pectolytic enzymes	-	-	-	-
Syringomycin	-	-	-	-
Ice nucleation	+	-	+	-
Growth factors required	-	-	-	-
Catalase	+	+	+	+
Indole	-	-	-	-

Testing in vivo. The trait of greatest interest to us is the ability to colonize plant surfaces, particularly strawberry blossoms. The INA+ and INA- strains demonstrated generation times of ca. 2 hours when sprayed onto newly opened strawberry blossoms and incubated under intermittent mist in a greenhouse at 21 ± 3 C (Figure 5). Mutant and parental strains also were able to colonize blossoms of almond, cherry, pear and blackberry and leaves of strawbery, bindweed and purslane. None of the strains colonized leaves of tomato, potato, tobacco, cotton, lambsquarters, yellow sweetclover or annual bluegrass. In soil and strawberry root environments the INA- bacteria exhibited poor fitness compared to other soil inhabiting bacteria, as did their parental strains. All of the above evidence suggests that the INA- strains may be as ecologically fit as their parents, but probably are not more so. Further, the INA+ and INA- strains exhibited identical properties when grown on strawberry blossoms in competitive situations with other bacterial strains. Competition between the isogenic strains was reciprocal in nature, and therefore the interaction was dose dependent (23). Thus, ice nucleation activity appears to have no influence on either axenic growth or competition between strains under optimal conditions. These data, combined with the physiological data from diagnostic tests have led us to conclude that the INA- strains indeed are not capable of propagating an imbalance in the ecosystem because they act exactly like wild-type strains. We expect the INA- strains will be effective competitors at

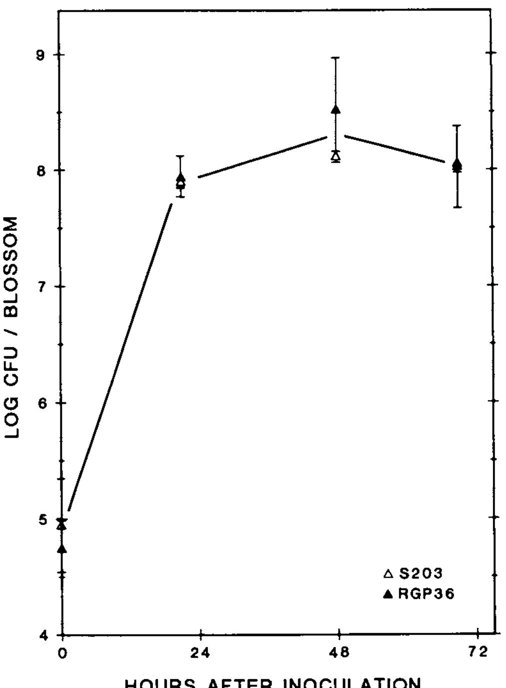

Figure 5. Comparison of the colonization potential (on strawberry blossoms) of the P. syringae wild-type strain S203 and its INA$^-$ deletion mutant RGP36.

sites where we apply them in large numbers, but not at distant locations to which they will be disseminated in low concentration.

Future Directions

Protection of strawberry blossoms from frost injury after exposure to a temperature of -3.5 C has been achieved under laboratory conditions when the INA$^-$ bacteria were challenged individually with several different strains of INA$^+$ bacteria. Similar results were previously reported with both chemically-derived INA$^-$ and deletion mutant strains of *P. syringae* on crops such as corn, pear and tomato (6,24,25). The next necessary step is to conduct field efficacy trials. Initially, a single trial will be conducted primarily to evaluate the environmental fate of the introduced INA$^-$ strains. The location and small size of this plot will limit its usefulness as an efficacy trial per se. However, after the environmental fate issue of this release has been evaluated under field conditions, larger trials will allow us to examine the effectiveness of the INA$^-$ strains applied to strawberry plants in commercial fields at several locations. Finally, efficacy trials could be expanded to include other major rosaceous fruit crops whose acreages are exposed to frost hazards.

Following the trials in commercial strawberry fields, we may wish to initiate laboratory efforts toward strain improvement for increased efficacy. This would be expedited by an understanding of the mechanism(s) of growth inhibition of the INA$^+$ by the INA$^-$ strains. Research aimed at elucidating such a mechanism is currently in progress. In the future, efforts to improve efficacy might involve 1) screening for variants with faster growth rates at cold temperatures, 2) introducing an additional inhibitory characteristic (eg. antibiotic production) or 3) introducing a selectable trait (eg. copper resistance) so that biological and chemical controls could be combined in an integrated pest management program.

Government Regulation of the Research

The legal aspects of using ice nucleation deficient bacteria for frost protection are responsible for the attention accorded to this research by the popular media. Therefore it seems appropriate to provide a scientists' perspective on these issues and on the regulatory process as it applies to deliberate release experiments.

The novel interest here was that the field tests proposed would have involved the first *deliberate* release of genetically engineered bacteria. In the United States, the National Institutes of Health (NIH) already had established a Recombinant Advisory Committee (RAC) to consider the hazards of *accidental* release; it was natural that RAC should assume responsibility for regulating the new class of experiments. The RAC guidelines show that the accidental release of engineered *P. syringae* is not considered a serious problem, since the construction of such strains is permitted under low levels of physical containment. The key difference between accidental and deliberate release is in the number of organisms likely to be introduced into the environment: thus the main question was whether

the dispersal of large numbers of engineered bacteria could cause significant environmental impact beyond the field plot where they were to be applied (in the case of our experiment, an area of less than 0.5 acre).

In 1982, S. Lindow and N. Panopoulos applied to RAC for permission to release ice nucleation deficient bacteria; permission was denied because of their initial plan to replace the ice nucleation gene with an element conferring resistance to the antibiotic kanamycin. Their second application was approved but legal constraints prevented execution of the field test. Similarly, legal considerations at first delayed the review of our own 1983 application, and subsequently prevented the NIH director from acting on the RAC recommendation to confirm its approval. In 1984, the Environmental Protection Agency (EPA) determined that ice nucleation deficient bacteria should be considered a form of pesticide. This classification will probably apply to most bacterial products with agricultural applications, but not to other types of recombinant organisms. The existing procedures for regulation of pesticides dictated that an experimental use permit (EUP) would be required for field testing of the bacteria. The EPA is responsible for granting an EUP, basing their decision on a review of submitted data. Approval is also required from the appropriate State authorities. At the time of writing, it is reported that NIH-RAC will withdraw from the role of regulating deliberate-release experiments.

Implications for Agriculture

The principle underlying our approach to frost protection is to use closely-related organisms as competitors against pathogenic microbes. This idea is not novel but as explained above, its most satisfactory testing requires genetic engineering of the competitors. Therefore this approach is liable to yield some new information with wider implications for agriculture. Much has already been written, both in enthusiasm and in caution, about the directions in which deliberate release experiments are leading. We will confine the discussion to its definable, scientific implications. These will be limited to the other cases in which the "sibling competition" principle could be expected to operate. Therefore:

1) A partial, rather than a complete, reduction in the population of the pathogen must be capable of yielding significant results.

2) The colonization and competition ability of the pathogen must be independent of its ability to cause disease (else non-pathogenic derivatives will not be effective competitors).

3) Alteration of only one or a few genes must be capable of eliminating pathogenicity.

4) The pathogen must exist at sites which are accessible to some form of delivery system for the competitor.

5) The pathogen must exist at population levels where intercellular competition is the main or only factor limiting its population.

Epiphytic ice nucleating bacteria provide an ideal system for studying the feasibility of "sibling competition". All the above conditions are thought to be met, except that no. 5 is demonstrated

in the laboratory only. It is the major unknown in the field experiments still to be done. If it holds true in the field, many other types of bacterial disease may be controllable in analogous ways. Other diseases caused by epiphytic bacteria will be the best targets (26), although the same principle is potentially applicable to bacteria that live inside the plant or in its rhizosphere.

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Chapter 19

Immunoassays for Crop Management Systems and Agricultural Chemistry

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Immunoassays are analytical procedures based upon the specific binding of animal-derived antibodies to a target molecule. When coupled with a visualization method, immunoassays provide simple, specific and sensitive detection and quantification of a very broad range of analytes. Many medical diagnostic procedures and products, including many home diagnostics, are based upon antibody reagents. Agricultural analyses are a natural extension of this technology. Targets include plant pathogens, pests, pesticides, crop components, nutrient markers, and genetic markers. Immunoassays will be applied as informational tools at many levels of crop production to guide management of crop inputs. Rapid analysis of pesticide residues, early detection of plant diseases, and quantification of pest levels will provide information useful in determining crop rotation patterns, variety selection, pesticide selection, pesticide application timing, harvest dates, post harvest handling and many other management aspects.

Of the many disciplines within "biotechnology", the field of immunochemistry has had the earliest commercial impact primarily through the development of diagnostic products for the human health care industry. Highly effective immunoassays have become powerful tools in the analysis of such diverse materials as therapeutic drugs, drugs of abuse, microorganisms, tumor markers, and hormones. Such assays are routinely employed in clinical laboratories and, in recent years, have been moved into doctors' offices and the home. The simplification of test formats, availability of electronic instrumentation, and the development of monoclonal antibody techniques have stimulated rapid growth in the medical diagnostics industry over the past 10 years.

Immunoassays are based on the ability of biological molecules, antibodies, to bind very specifically with other molecules. When the binding is measured or visualized in some way, it becomes the basis for a simple, specific and sensitive assay. Antibodies display remarkable specificity and bind only to a

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narrow range of target molecular structures and configurations. Vertebrate immune systems are equally remarkable in that they can produce specific antibodies which recognize and bind to a virtually unlimited range of molecular structures.

These two characteristics of immune systems- (1) the ability to produce antibodies to an almost unlimited range of targets and (2) the ability of antibodies to recognize and bind tightly to a specific molecular configuration- permit the development of unique analytical procedures. Immunoassays differ from other analytical methods in that specificity is conferred by the reagents employed rather than by an apparatus of some type. Sensitivity is conferred by the strength of the antibody:antigen interaction and by the visualization method employed to detect the binding.

Agricultural research and development and crop management require vast amounts of analytical data that provide information upon which key decisions are based. Toxicity values, chemical residue levels, nutrient levels, and insect counts are representative of the diverse array of analytical information that is directly utilized by modern agriculture. Despite their wide use in medicine, immunoassays have been utilized in crop agriculture primarily as research tools in a limited number of laboratories. Relatively few commercial products based on immunoreagents are intended for detection of agricultural chemicals or other targets of agricultural interest. However, many of the same factors that favored the development and widespread use of immunoassays in medicine over the past 20 years are now at play in the agricultural arena. Such assays can be expected to play an increasing role in all aspects of crop management over the next 10 years. The applicability of immunoreagents to the rapid and cost-effective detection of a broad range of targets is very appealing for use in many crop management systems.

This review briefly examines the basis of immunoassay as an analytical method. It outlines current and future applications of these methods to detecting materials of direct importance to agricultural chemistry and crop management.

The Technology

The technology which has enabled scientists to develop immunoassay detection systems is based on unique properties of the naturally occurring defense mechanism of higher vertebrate animals, the immune response. The immune response consists of a series of events which enable the body to recognize foreign organisms or substances and to respond against these agents in a protective manner. The portion of the immune response utilized in immunoassay technology is that involving production of proteins called antibodies. Antibodies are produced as part of the humoral immune response when foreign materials are introduced into the body of an animal. Antibodies then bind very specifically to the foreign substance, destroy or neutralize it, and help clear it from the system. Antibodies recognize specific portions of a foreign substance, the binding partner allowing immunoassays to distinguish between that binding partner and similar substances and to detect small amounts of the target substance.

Antibodies are produced for use in an immunoassay by exposing

an animal or specialized cells from an animal to a target substance. For example, a laboratory animal such as a rabbit may be immunized with a preparation of the target substance to stimulate the production of antibodies. Larger animals such as goats, sheep or horses are commonly used to produce larger batches of antibodies. For the production of monoclonal antibodies, mice are immunized and antibody producing cells from the mice are utilized in the creation of the antibody producing "hybridoma" cells. More recently, cells with the capacity to produce antibody have been isolated from the animal and their growth established *in vitro* before they are exposed to the target substance. This procedure has been particularly important (for obvious reasons) in the production of human monoclonal antibodies (1). The antibodies are then incorporated into an immunoassay which can be used to determine whether that target substance is present in a test sample.

Basic Principles of Vertebrate Humoral Immunity. Physical barriers such as the skin and mucous membranes provide an animal's first line of defense against organisms or other substances in the environment. When the physical barrier is broken, the immune response is largely responsible for eliminating or inactivating foreign substances or organisms. The immune "surveillance" system recognizes foreign substances that are introduced into the animal as "non-self". This recognition activates the immune response. The immune system is comprised of a network of specialized cells and tissues which, when activated, produce various molecules. These molecules, which include antibodies, protect the body from harm caused by foreign agents by neutralizing or destroying them.

The immune system normally responds only to foreign or non-self substances. When the immune system fails to recognize a substance as foreign or responds slowly to the substance, the result may be life-threatening illness or infection. Sometimes the immune system loses its ability to distinguish between self and non-self, and a response is directed against "self" tissues. Diseases resulting from this immune malfunction are collectively referred to as autoimmune (against "self") diseases. Examples of autoimmune disease include rheumatoid arthritis and systemic lupus erythematosis. The immune surveillance system also has the ability to detect altered composition of self tissues so these tissues or cells can be destroyed. When altered self tissues are not detected or effectively destroyed, cancer may result. Clearly, the immune response is an intricate system requiring a number of complex functions to be effective.

The Immune Response. The immune response of a vertebrate animal has two major arms, the cell-mediated responses and antibody-mediated or humoral responses. The cell-mediated response, which is involved in protection against cancer and some intracellular pathogens (2), will not be reviewed here because it is not relevant to most immunoassay technology. The humoral response, which involves production of antibodies to foreign substances, is the arm of the immune system which provides the basis for immunoassay systems.

The humoral immune system can respond to foreign substances

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such as viral, bacterial and fungal pathogens as well as chemicals or particulates which gain access to the body. Any substance which triggers the production of an antibody and binds to that antibody is called an antigen. Current theory postulates that antibodies can be formed against antigens contained in virturally all materials.

In the reticuloendothelial system of animals, various cell populations cooperate to produce the humoral immune response. These cells circulate throughout the body in the blood and lymph systems. They are found in especially high concentrations in the spleen and the lymph nodes. Foreign antigens are initially recognized by scavenger surveillance cells called macrophages. Some substances are engulfed and destroyed by macrophages. Macrophages process the degraded antigens then "present" these antigens to other cells called T and B lymphocytes. The T lymphocytes have antigen-binding receptors. When specific antigens bind these receptors, the T-cells produce signal molecules which help activate or boost the activity of both macrophages and B-cells. B lymphocytes also bind the specific antigens presented by macrophages. B-cell responses are enhanced by signal molecules from macrophages and T cells. As a result of antigen recognition and molecular signaling, B-cells are induced to differentiate into plasma cells. The plasma cells then produce antibodies that bind to the antigen which triggered this series of events. Each plasma cell produces antibodies of one specificity. That is, they bind to a small but specific region of the inducing antigen called the antigenic determinant. Different plasma cells produce antibodies specific for different portions of the foreign substance. This results in the overall production of antibodies which bind to different sites on a given substance. This is referred to as a polyclonal antibody response. Antibodies produced during the humoral response are secreted by plasma cells and are found in high concentrations in the blood stream.

The humoral response is complex, and several days are required before antibodies are produced against a foreign substance or antigen that enters the body. When animals are purposely exposed to a substance to stimulate antibody production, several weeks of repeated exposure to the antigen may be required before high levels of specific antibody are present in the serum.

Immunoglobulins - Structure and Function. Antibodies are globular proteins produced during the immune response and consequently, they are also called immunoglobulins (Ig). Porter and Edelman won the Nobel prize in Physiology and Medicine in 1972 for their fundamental research on the structure and chemistry of Ig (3). The classes of Ig produced by humans include Ig A, G, E, M and D. Antibodies of different classes range from 150 to 900 Kilodaltons (Kd) in molecular weight and mediate different immunological functions. Some other vertebrate animals produce fewer classes, but most produce IgG and IgM. These two antibody classes are found in serum and may be readily used in immunoassays. IgA is also used in some cases.

An IgG molecule consists of two heavy chains and two light polypeptide chains connected through disulfide bonds (Figure 1). IgG antibodies have a molecular weight of 150 Kd and account for approximately 80% of the total immunoglobulin in human serum.

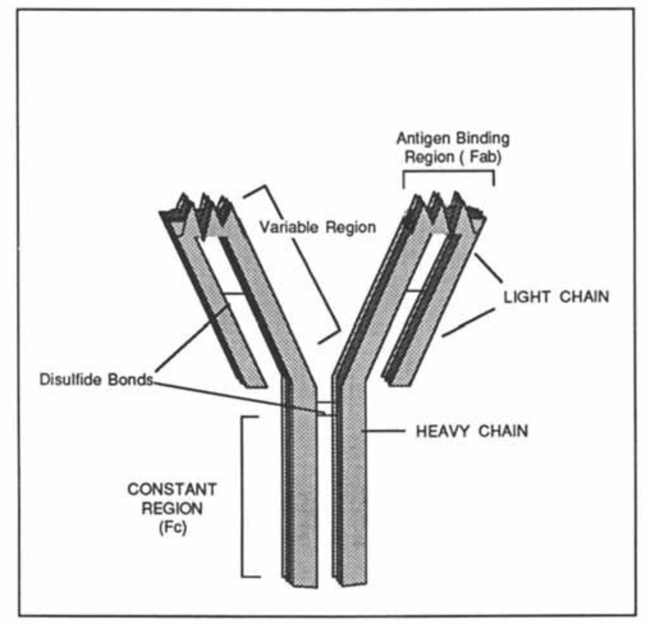

Figure 1. IgG antibody molecule consists of four chains, two heavy and two light, held together by disulfide bonds. Antibody binds to antigens as a result of the molecular configuration of the variable region.

The Fab portion of the antibody (see Figure 1) contains the variable region that is responsible for the specificity of the molecule. Variations in the polypeptide sequence of this region are complimentary to the antigenic determinent thus providing the basis for antigen:antibody binding. An IgG molecule has two antigen binding sites.

The Fc region of an antibody molecule (see Figure 1) is constant within a particular antibody class. The Fc region mediates secondary immunological functions such as complement fixation (4). Labels or "tags" which are used for visualization of immunoassays are generally attached to the Fc region so the antibody retains the antigen-binding capacity of the Fab region.

The polyclonal nature of the humoral response results in the production of antibodies with different binding specificities. In addition, antibodies vary in binding affinity, or strength of antibody-antigen binding. Polyclonal antisera exhibit a range of specificity and affinity within a given pool of serum.

Immunoassays. Immunoassays are tests in which antibodies are used as analytical chemistry reagents. The immune response of an animal can be manipulated to produce antibodies that react with a particular target antigen. Serum from the blood of the animal, which contains the antibodies, can then be collected at the appropriate time. Finally, the antibodies are purified and incorporated into a detection system. The key to successful development of an immunoassay lies in producing tests that provide accurate information; that is to say, tests with high levels of specificity, sensitivity and reliability. Diagnostic sensitivity means that a test is positive whenever the target antigen is present, even at low levels. Equally important is specificity, where the test is negative when the target antigen is not present. The combination of specificity and sensitivity are used to determine the predictive value of a positive or negative test, or the percent reliability of the test.

Reliability of an immunoassay is essential to its success in the market. The consumer may then benefit from their use by being able to take timely action. The rapid advances in biotechnology and immunology over the past decade have made it possible to improve both the specificity and sensitivity of immunoassays.

To a large extent, the antibody is responsible for the specificity and sensitivity of the immunoassay. Antibodies suitable for use in some immunoassays may be produced by immunizing a laboratory animal with a relatively complex preparation containing the antigen to be assayed. Such antigens may be component parts of microorganisms such as bacteria, viruses, fungi, protozoa, or helminths. Antigens may also be chemicals, plants, or plant products. Additional materials called adjuvants are commonly injected in conjunction with the antigen-containing preparation to enhance the production of antibodies.

Some molecules are too small to elicit an effective humoral response. These molecules, defined as haptens, must be coupled to a larger protein before being introduced to the body, in order to elicit an antibody response to the hapten. The larger molecule,

known as a carrier, helps produce the necessary recognition signals for activation of the immune response. This type of system is employed to produce antibodies which can then bind to certain chemicals and to small polypeptides (5, 6).

Optimal immunization schedules (number of injections and time between injections) vary widely depending upon the characteristics of the particular antigen. Serum, which contains the antibody, is collected from the animal and used in the immunoassay. Antibodies produced by immunizing an animal and collecting the antibodies directly from the serum of the animal are referred to as polyclonal antibodies. Polyclonal antibodies exhibit a range of specificities and affinities within a given pool reflecting the complexity of the preparation which was injected into the animal. Often, a more useful polyclonal serum can be produced if the most desirable antigenic component(s) can be identified and purified before the preparation is injected into the animal. For example, when a whole organism is injected, antibodies with hundreds of specificities are produced. Although some of the antibodies are specific to the injected organism, many of the antibodies are likely to also react with related organisms and some antibodies may even react with unrelated organisms. This problem can sometimes be eliminated by immunizing only with an antigen that is unique to the target organism. Obviously, this approach may require a considerable amount of analytical and preparative work before the immunizations are initiated.

Commercial quantities of polyclonal antibodies can be obtained simply by immunizing large numbers of animals. Even under the best of circumstances, however, lack of reproducibility may be encountered because of considerable differences in the response of individual animals to an identical antigen preparation. Some of this difficulty may be overcome by using large animals such as sheep, goats, horses or by pooling sera to produce large batches or lots.

Monoclonal Antibodies. One of the most useful, recent scientific advances to be applied to immunoassay technology has been the development of monoclonal antibodies (7). This technology was first reported in 1975 by Kohler and Milstein (8), who won the Nobel Prize for their work. Although much of the current application of monoclonal antibody technology is related to human medical research and diagnostics, veterinary and agricultural applications are expanding rapidly (9).

Monoclonal antibodies are produced in a series of steps beginning with the immunization of a mouse and removal of its spleen after an appropriate period of time. Antibody-producing cells are isolated from the spleen and fused with "immortal" myeloma cells from tissue culture through the use of polyethylene glycol. Cells resulting from the fusion of a B-cell and a myeloma cell are called hybridomas. Hybridomas derive the ability to produce a single type of antibody from the B-cell partner and the ability to survive and proliferate outside the body of the animal for an extended period of time from the myeloma cell partner. Through a series of manipulations in tissue culture, individual hybridomas are isolated and allowed to divide and produce antibody. Antibody in the cell culture medium is then tested for

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desirable antigen-binding characteristics. Since each hybridoma cell line produces only one type of monoclonal antibody, selection of an appropriate hybridoma line makes it possible to avoid many of the potential problems encountered with antibodies in polyclonal sera. Hybridomas may be frozen in liquid nitrogen, stored and recovered at any time to continue reproducible production of antibody.

Monoclonal antibody production is "scaled-up" either by growing large numbers of cells in tissue culture or by injecting the cells back into the peritoneal cavity of a mouse to produce antibody-rich ascites fluid. Tissue culture scale-up procedures utilize a variety of bioreactors, hollow-fiber filters, re-circulating media and cell encapsulation technologies designed to promote rapid growth of the cells and increase the quantity and purity of antibody produced. Ascites production takes advantage of the fact that the best environment for growth of the hybridoma cells and production of antibody is probably in the animal host species from which the cells originated. Ascites fluid contains a much higher concentration of antibodies than tissue culture supernatants. However, the quantity and complexity of contaminating materials is also greater in ascites fluid.

Antibodies must be purified from the tissue culture supernatant, ascites fluid, or serum before they can be "tagged" or otherwise effectively utilized in an immunoassay. Purification of antibodies can be accomplished to varying degrees employing a number of physical, biochemical and immunological techniques (10).

Visualization/Detection of Antibody/Antigen Interactions. For practical application of the unique properties of antibodies in a diagnostic assay, the user must be able to determine whether the antigen/ antibody reaction has in fact occurred. A variety of visualization and detection methods have been designed for this purpose.

Precipitation/Agglutination. One of the oldest techniques for visualization of antigen-antibody reactions is through the observation of a precipitation or agglutination reaction. All antibodies and some antigens are multivalent (have more than one binding site). A lattice or matrix is formed when the antibodies react with the antigens. In some cases, the lattice is large enough that it is visible to the naked eye. The precipitation reaction can be carried out in solution (turbidity test or ring precipitation) or in a gel matrix (Ouchterlony double diffusion or radial immunodiffusion). Precipitation reactions in gels can be enhanced by staining. Reactants can also be attached to particles such as red blood cells or latex beads to make the reaction easier to visualize. Precipitation/agglutination assays are extremely simple to perform but the results can be difficult to interpret at low concentrations of the target reactant.

Complement Fixation. Complement is a component of the immune system capable of binding to an antibody after the antibody has reacted with an antigen. If that antigen is part of a whole cell, complement mediates a series of events which results in the lysis of the cell (11).

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A complement fixation assay uses red blood cells and antibody to the red blood cells as an indicator system (see Fig. 2). An antibody produced against the target antigen is reacted with the unknown test sample. If the target antigen is not present in the test sample, no antigen:antibody complex is formed by the test system and complement is not "fixed" by the test system. The complement subsequently lyses the red blood cells when the indicator system is added. If target antigen is present in the test sample, the antigen:antibody complex in the test system ties up the complement, and the red blood cells are not lysed when the indicator system is added. The degree of lysis of the indicator system is determined by observing the supernatant of the reaction mixture. Hemoglobin from the red blood cells colors the supernatant red if the red blood cells are lysed.

Enzyme Immunoassay. Enzyme immunoassay or EIA is probably the immunoassay technology most widely applied today (12). In an EIA, either the antibody or the antigen is tagged with an enzyme. After the assay is performed, the presence or absence of the enzyme-labeled component is detected by the addition of an appropriate substrate. EIA's are quite sensitive, reagents are relatively inexpensive and widely available, and the reagents are relatively stable. Quantitative results can be obtained using instrumentation and EIA is easily automated.

Immunoassays which require that unbound reactants be separated from the bound reactants (usually by washing) before the signal is generated or detected are referred to as heterogeneous immunoassays. One popular configuration of a heterogeneous EIA is illustrated in Figure 3. This format is not frequently used for evaluating unknown samples because of the possibility of interference from irrelevant molecules in the sample preparation. The format is widely used for screening of monoclonal antibodies and evaluating polyclonal antibodies in situations where a relatively pure preparation of the target antigen is available. The target antigen is first attached to the solid support such as a 96-well microtiter plate and excess antigen is washed away. The test antibody is then incubated in the antigen-coated well. After an incubation period, the unreacted antibody is washed away. An enzyme-tagged antibody made to react with the immunoglobulin species of the test antibody is the next reagent placed in the well. A final wash step is performed and colorless substrate is added. Generation of color by the substrate (mediated by the enzyme) is indicative of the presence of an antibody which reacted with the target antigen.

For evaluation of the amount of a target antigen present in an unknown sample, a pure antigen preparation is bound to the plate. The test sample is combined with measured amount of antibody which is known to react with the target antigen. The sample antibody mixture is then incubated in the antigen coated well and the quantity of antibody bound to the well is subsequently determined as previously described. This format is generally referred to as a binding-inhibition assay because if antigen is present in the test sample, the antibody is depleted by the test sample and is not bound to the antigen on the plate. In this case, the generation of color in the wells would indicate that the target antigen is *not* present in the test sample.

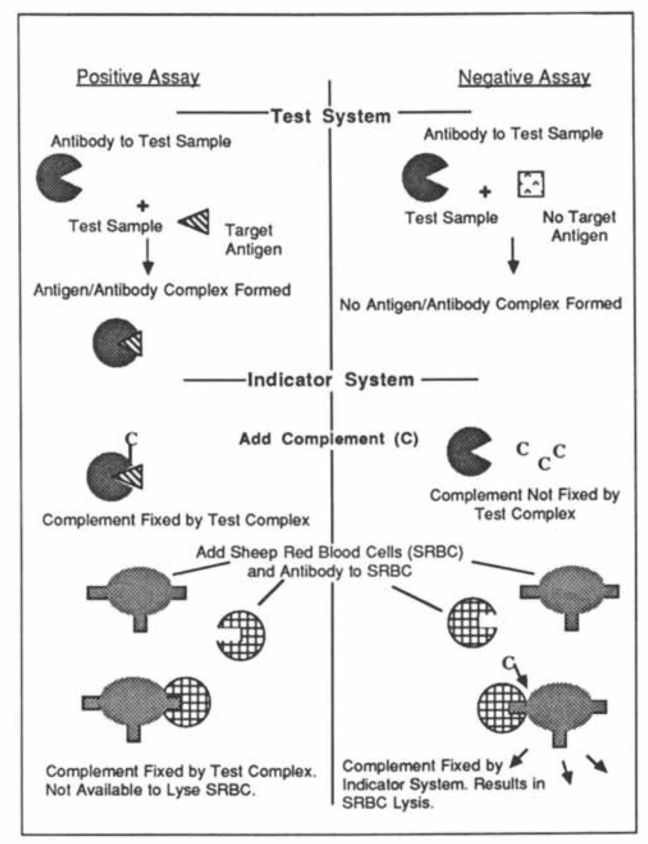

Figure 2. Complement fixation immunoassay. Red color released due to the lysis of red blood cells indicates a negative assay, i.e., the absence of the target antigen.

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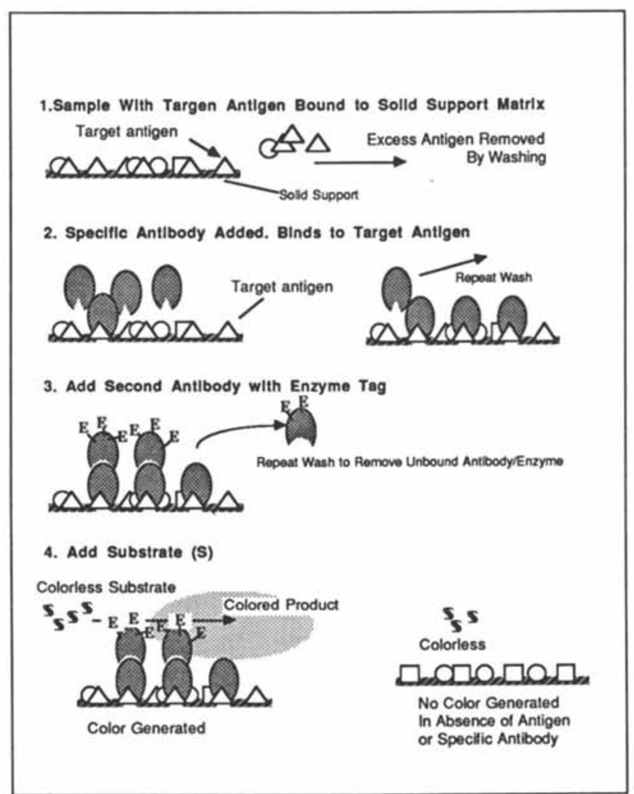

Figure 3. Enzyme linked immunosorbent assay (ELISA) represents a heterogeneous assay in which unreacted materials are washed away between steps. Antibody-antigen binding is visualized by the generation of a colored product.

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Another useful heterogeneous EIA is the double antibody or "sandwich" immunoassay. In a sandwich immunoassay, an untagged antibody or capture antibody is the first reagent bound to the solid phase. The test sample and then the enzyme labeled antibody are added sequentially. The unbound components are removed by washing after each incubation. Enzyme-labeled antibody is detected by addition of a substrate as above. The capture antibody is particularly useful when the test sample consists of a complex mixture of materials. The capture antibody "fishes-out" the target antigen and other components of the test sample (which could interfere with subsequent steps in the immunoassay) are more effectively washed away.

Sensitivities of many EIAs can be increased through the use of avidin-biotin amplification systems (13). Avidin is a 68 kd glycoprotein which can be purified from egg whites or from some species of bacteria. Biotin is a small molecular weight vitamin. Avidin binds biotin in an essentially irreversible reaction. The affinity of this reaction is over one million times higher than the affinity of antibodies for antigens. In one example of an amplified EIA, antibody prepared against the target antigen is tagged with several molecules of biotin. The enzyme is bound to avidin. The high affinity reaction between avidin and biotin and the fact that each avidin molecule has four binding sites for biotin amplifies the number of enzyme molecules present for each bound antibody molecule. The larger number of enzyme molecules present generates a stronger signal when substrate is added.

An immunoassay that is extremely useful in the detection of small molecular weight compounds (i.e., pesticides, drugs) is the homogeneous enzyme-immunoassay. In a homogeneous immunoassay there is no need to separate the bound reactants from the free reactants before the signal is measured. In order to develop a homogeneous immunoassay, the antigen to be detected must be obtained in pure form. Enzyme is covalently bound to the antigen in such a position that the enzyme activity is sterically altered if the appropriate antibody binds to the tagged antigen (see Figure 4). To perform the assay, the enzyme-tagged antigen and then the antibody are added to the test sample. The final result is then obtained by the addition of substrate which can react only with enzyme in its uninhibited form. A variety of other useful configurations for EIAs have been demonstrated depending on the particular application (14).

Other Detection Systems for Heterogenous Immunoassays. Tags other than enzymes which have been used successfully in heterogeneous immunoassay systems include radioisotopes, fluorescent dyes, and colloidal gold. Similar assay procedures are performed using these tags, although different detection methods are employed to determine the result of the assay.

In an immunoflorescence assay, fluorescent dyes such as fluorescein and rhodamine can be visualized on a microscope slide through the use of ultraviolet light or detected with instruments which measure their emissions. Fluorescent immunoassays are widely used in clinical laboratories. They are quite sensitive but may be time-consuming and reagents may be unstable and require special handling.

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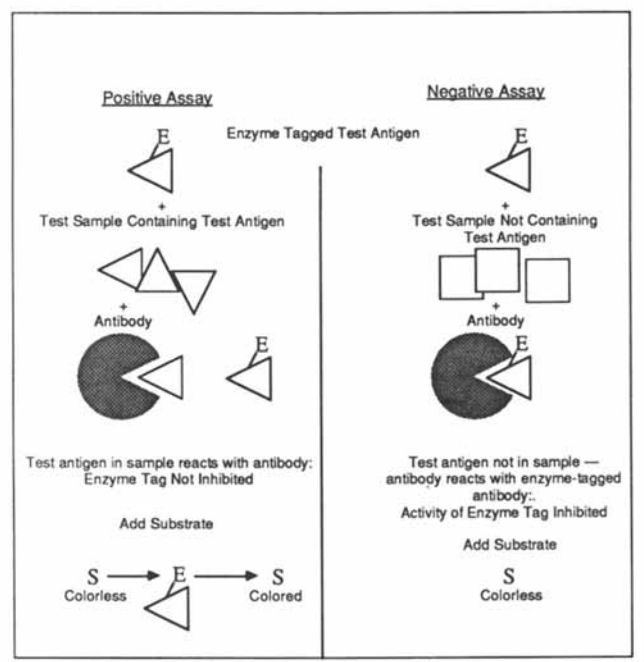

Figure 4. Homogeneous immunoassay in which results are obtained without washing procedures. The assay depicted is competitive - unlabeled antigen in sample competes for antibody binding site with enzyme labeled antigen.

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Radioisotopes have been widely used an indicators in assays that are appropriately referred to as radioimmunoassays (RIA). Yalow won the Nobel Prize for work leading to the development of RIA (15). RIA's typically have a high degree of sensitivity and reproducibility. Additionally, automated instruments have been designed that make it possible to process a large volume of samples. RIAs are also very popular in clinical microbiology laboratories. Drawbacks for RIA's include the need for expensive equipment to read the radioactive signal, short half life of some labels, potential health hazards to personnel, and waste disposal problems.

Colloidal gold labeled antibodies are useful when the immunoassay is performed using an affinity membrane or paper (such as nitrocellulose) as the solid support matrix. The color generated from a colloidal gold tagged reactant can be visualized directly. The sensitivity of the immunoassay can be increased by an additional step during which the gold is reduced and generates additional color (16).

Assay Formats. Recent modifications in the actual formats used for the execution of immunoassays have generally been designed to make the tests faster and more convenient to perform and interpret. Most commercial diagnostic applications are changing from laboratory assays which require trained technical personnel and expensive instrumentation to assays that can be performed in the doctors office, in private homes or in the field.

Originally, most immunoassays were performed in test tubes, on microscope slides or in 96 well micro-titer plates. This "solid phase" component of the immunoassay has been replaced by a variety of paraphernalia designed to make the test easier to perform and interpret and to make the assay compatible with the many different types of samples currently being analyzed.

The basic technologies used to detect antigen in the sample are unchanged or only slightly modified from the previously described techniques in most of the new immunoassay "kit" formats. A large variety of executions have been developed to fill the increasing and diverse demand for immunodiagnostic assays. The key components of some of the more popular commercial immunodiagnostic kits are described below.

Immunoassays can be performed on the surface of beads or other particles. The composition and size of the beads may vary depending on the application. Surface area available for the reactions is greatly increased by using beads instead of a flat surface as the solid phase. Additionally, beads can be easily transferred from one reagent to another and washing steps are rapid and convenient (e.g. beads containing metal can be transferred magnetically).

One of the simplest to use executions of the immunoassay uses a dipstick as the solid phase. The dipstick is moved from one reagent to the next and the assay result is then determined by examining the dipstick. The color reaction in many EIA-dipstick assays is produced when an enzyme label converts the substrate into an insoluble colored product that binds to the dipstick. Semi-quantitative results can be obtained by comparing the color

intensity caused by the test sample to color intensity caused by known standards. Quantitative results can be obtained through the use of an inexpensive field adaptable reflectometer which measures the color intensity on the dipstick.

Dry chemistry technology eliminates the need for reagents in liquid solutions. Some reagents are immobilized in layers of porous material and dried. The immunoassay is performed by allowing the sample solution to migrate through the reagent layers where the reactions take place in the order in which the reagents have been layered. Migration of the sample solution generally takes place by capillary action. In some layered assays, force is applied in order to cause a specific layer of reagent to come into contact with the sample. It is possible to obtain semi-quantitative and quantitative results by using methods similar to those described for the dipstick assay.

The Applications

Commercial immunoassays are used in great quantities in medical chemistry laboratories to provide a wide range of information used by the physician in managing patient care. Diagnostic tests for infectious diseases, tests for infectious agents in blood supplies, assays for markers of specific diseases, and procedures for detecting drugs and other small molecules are routinely available. Immunoassay procedures for the doctor's office are becoming common and are allowing routine analyses to be performed on-site by the primary care physician. Testing kits targeted for use by consumers at home are also being marketed at an increasing rate. Most notable are home pregnancy test kits and kits for detecting Streptococcus in throat swabs.

Agriculture has analytical targets analogous to those described in the medical areas. Consequently, the utility of a new analytical procedure such as immunoassay is readily apparent. However, several factors have slowed the development of antibody-based assays for agricultural applications. The principle factor is the nature of the matrix to be analyzed. In medical immunoassays the matrix is usually blood serum or urine from a single host species - humans. Agricultural chemists and biologists deal with a wide variety of matrices ranging from relatively simple ground water to complex soils and macerated crop samples. Crop varieties, crop species, and widely variable environmental conditions add layers of complexity that are not routinely encountered in the clinical laboratory.

Despite the complexity of agricultural systems, immunoassays are being increasingly exploited because of their attractive characteristics of speed, relative low cost, and simplicity. The sections below outline applications of these tools for experimental use and for direct application to crop production.

Detection and Diagnosis of Crop Diseases. Diagnosis of infectious diseases of crops is one of the near term uses of immunoassays in agriculture (21). Effective management of plant diseases requires that crop managers have access to timely and accurate information on threats to their crop. Existing plant disease diagnostic procedures require trained personnel, well-equipped laboratories,

and a good deal of time. Diagnosis may require a few days to several months, depending on the nature of the pathogen to be detected.

Immunoassays offer simple, rapid, and sensitive detection of plant pathogens and pests in crops, soils, and other matrices. Plant virologists pioneered the use of antibody-based assays for plant viral disease diagnosis (17). Diagnosis of these plant virus diseases is particularly difficult because the viruses can not be cultured, and other diagnostic tests such as local lesion assays and electron microscopy are time consuming, labor intensive and/or require expensive equipment. Since plant viruses are distinct entities with a fairly well defined structure they could be purified and used as antigens to induce fairly specific polyclonal antibodies. Virologists could use these antibodies to detect viruses in plant extracts for rapid diagnosis.

The complex nature of fungi, bacteria, and nematodes made it difficult to develop specific antibodies that could be used for diagnostic purposes. The advent of monoclonal antibody technology stimulated interest in developing diagnostic reagents for these complex plant pathogens. The ability to screen a battery of monoclonal antibodies to identify those which recognize the target pathogen is an appealing feature of this technology since the biochemical composition of most plant pathogens and pests is not well understood. Subsequently, the specific antibodies can be used to characterize differences among pathogens at the genus, species, and even race levels. While the bulk of work in this area reported to date has involved plant viruses, monoclonal antibodies have been produced against plant pathogenic bacteria, spiroplasmas, mycloplasma-like organisms, and fungi (17, 19, 20). The beneficial characteristics of monoclonal antibodies are especially applicable to plant disease diagnostics due to the complexity of antigen preparations, difficulty in obtaining obligate parasites, and the lack of basic information on the biochemical structure of plant pathogens.

Diagnostic products based on antibodies will provide valuable information to guide many crop disease management decisions (18, 19). Some of the more important uses of immunoassays in detecting plant pathogens and pests include:

Rapid diagnosis of disease to confirm a visual diagnosis. This will be most useful when symptoms are sparse, undeveloped, or are present on roots. Because of the specificity of the antibody reagents used, primary pathogens can be detected even if secondary organisms have invaded the tissue. Multiple infections in which more than one pathogen is present can also be detected.

Presymptomatic detection of infection in which active infections are detected before secondary spread and serious economic loss are sustained.

Detection and quantification of pathogens will allow determination of pest and pathogen levels in soil, planting media, water and propagating materials such as seeds and cuttings. Levels can then be related to future

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yield loss so that appropriate management measures can be employed.

The use of the information generated with immunoassays is the critical factor in determining the ultimate utility of this technology in agriculture. Crop management decisions which may be influenced by specific information on pest and pathogen levels include:

> Crop rotation patterns based on the presence or absence of threatening levels of pathogens or pests. Planting decisions for specific fields or groups of fields can be made with knowledge of pest populations and probable need for chemical treatments.
>
> Variety selection will be influenced similarly. For example, a high yielding but disease-susceptible variety might be planted if immunoassays indicate relatively low pathogen levels in a certain field.
>
> Choice of chemical treatment either at planting or during the growing season can be based on information on pest distribution, density, and identity. Such information can be used by the knowledgeable grower or crop consultant to select chemicals, use rates, and timing of application within the ranges approved for use.

Certification of seeds and other propagating materials as being disease-free or disease-tested is another short term application of immunoassays. Commercial products or services employing antibody-based tests are available for detecting viruses in seed potatoes and lettuce seed. These assays are performed primarily in central laboratories, but simplification of assay formats and procedures will permit seed crops to be monitored more closely throughout the production cycle to assure that elite stock remains pathogen or pest-free.

Diagnostic materials for field use are under development at a number of commercial concerns and research centers. Over the next 3-5 years several immunoassays in kit form will be available to crop managers to use as tools for managing agronomic practices. The manager will use information from diagnostic kits as a portion of the total information that goes into the complex judgements made in successful modern agriculture.

Detection and Quantification of Crop Protection Chemicals. Immunoassay procedures provide additional analytical tools for determining the presence and quantity of specific crop protection chemicals in various matrices. The specificity of an immunoassay is provided by the antibody reagent rather than by the analytical apparatus. The specificity, as noted above, allows the analysis of relatively crude preparations rapidly if the appropriate reagents (ie. antibodies) are readily available. Since a specific reagent must be prepared for each analyte, the greatest utility for these

assays lies in analysis of crop, soil and environmental samples for specific pesticides and a limited number of degradation products. Such assays can generally be conducted with much cruder preparations than are possible with conventional methods (HPLC and/or GLC) which results in time and cost savings.

One immunoassay method (22) for the insect growth regulator diflubenzuron was less than 10% as costly as HPLC or GLC, allowed 10-50 times as many samples to be analyzed in one day, and required lower capital costs (Table 1). Assay sensitivity in stagnant water and milk was at least equal to that obtained with the standard methods. The greatest time and cost savings resulted from the reduction or elimination of cleanup steps required. Other authors have reported similar results for a variety of pesticides analyzed with immunological assays (23, 24).

TABLE 1
COMPARISON OF HPLC, GLC, AND IMMUNOASSAY

Analyte: Diflubenzuron insecticide
Substrates: Stagnant Water, Milk

Method	Matrix	Clean Up Steps	Assays Per man-day	Labor: Sample	Reagent Cost per Sample	Sensitivity Limit	Sample Size
HPLC	Water	5	5	1.6 Hr	$4.00	10ppb	250ml
ELISA	Water	0	>50	5 min	0.20	1.0ppb	0.5ml
GLC	Milk	19	0.5	16 Hr	$11.00	50ppb	20ml
ELISA	Milk	0	>50	7 min	0.20	40ppb	0.2ml

Adapted from (22)

Immunoassays for pesticide analysis can readily be structured as laboratory analytical methods employing manual, semiautomated and automated formats. The key step in developing the analytical procedure is generating antibodies which recognize and bind to the proper chemical species. Target molecules must be covalently linked to carrier proteins in such a way that the characteristic portions of the target are free to bind with the variable regions of an antibody. It is necessary, therefore, that the exact structure of the target molecules and of non-target chemicals be known before antibodies are developed. One of the major differences between immunoassays and conventional analytical procedures, that immunoassays for small molecules are not generally suitable for detecting or determining the structure of unknown materials. Immunoassays may be useful for indicating whether an unknown substance shares some common structure with a known material for which an antibody exists, however.

Once antibodies to a specific chemical have been generated, they must be evaluated to determine the range of structural variation the antibodies recognize. Antibodies with differing levels of specificity can often be generated through careful design of the immunogen. For example, class-specific antibodies can be developed by leaving a common moiety exposed so that

antibodies that recognize features shared by a family of analogs can be selected. In many instances cross-reactivity is undesirable and antibodies must be developed which do not recognize closely related molecules. Many examples exist of the development of highly specific antibodies. Wie and Hammock (25) reported polyclonal antisera which differentiated the insected diflubenzuron and the chemically related compound, BAY-SIR 8514. In extreme examples, several workers have reported antibody reagents which distinguished optically active isomers and cis- and trans- forms of the target molecule.

Sensitivity of immunoassays for small molecules is generally comparable to that obtained with HPLC and GLC methods. As with all analytical procedures, several qualifying statements must be made regarding absolute sensitivity of an immunoassay. The level of analyte detected will vary with the substrate being examined, interfering substances, and the nature of the analyte and reagents themselves. Most immunoassays can be successfully performed on crude extracts of crop and environmental samples with no or minimal clean-up steps. In clinical use, highly precise and accurate analyses are routinely performed on patient samples with no sample preparation other than filtration and dilution. The key factor in this instance is developing an antibody reagent which has a high affinity for the analyte. The antibody:antigen (or analyte) reaction is reversible, consequently assay sensitivity will be proportional to strength of the binding and to the nature of the signal generated by the antigen:antibody complex.

The ability to perform quantitative assays on complex mixtures with little sample clean-up is perhaps the most attractive feature of immunoassays for application to agricultural chemistry. A large portion of the cost and labor involved in pesticide residue analysis is invested in sample extraction and clean-up steps to remove substances which may interfere with subsequent chemical analysis. Since most preparatory steps are not required prior to performing an immunoassay, samples can be analyzed much less expensively. This will permit the vast number of data points required for pesticide registration to be gathered in a more timely and cost-effective manner. Studies which were prohibitively expensive because they would have required large numbers of expensive assays can be completed using immunoassay procedures. Such studies may include analysis of pesticide movement from application areas and the rate of dissipation of pesticide from crop tissue, soils, and processed foods.

A second highly attractive feature of immunoassays is the speed of most procedures and the number of assays that can be completed in one day. Using semi-automated equipment coupled with a personal computer, a single person can generate 1,000 to 10,000 data points per day. Since the specificity of the analysis is provided by the reagent, the equipment used can be applied to many different assays. Again the only limitation is the supply of high quality antibody reagents.

Although antibody-based assays have many attractive features, we must emphasize that classical analytical methods must be used to confirm and validate immunoassays which are used for quantitative and/or qualitative analysis of specific molecular structures. Since most immunization procedures generate antibodies

to carrier proteins and linking moieties as well as to the target chemical, various conventional methods including MNR, IR, and mass spectoscopy must be employed to assure that the assay detects the target molecule.

Quantification of an analyte in an agricultural sample relates to both the assay procédure and the sample preparation procedure. Conventional analyses frequently focus on total residues and may employ relatively harsh extraction procedures to remove bound materials from the soil or crop sample. The biological nature of antibodies requires that most immunoassays be run in aqueous systems at a pH near neutrality. If total residues are to be measured and extraction requires the use of organic solvents, strong acids, or strong bases the extracted materials must be dispersed in an aqueous medium prior to using an immunoassay. Moderate levels of methanol (<10%) and other water miscible solvents do not interfere with most immunoassays.

Since immunoassays are aqueous, they are ideal for detecting and measuring biologically active residues. Aqueous extraction of soils followed by rapid immunoassay could provide timely data on potentially phytotoxic levels of herbicide residues or could determine whether insecticide levels had fallen below the biologically significant point. Data of this nature can be used by crop managers as they determine crop rotation patterns, pesticide purchasing and application plans, and reapplication timing. These analyses could be performed readily in soil nutrient laboratories, chemical company analytical departments, or simple laboratories at co-ops or chemical distributor companies.

Local Analysis. One of the most promising applications for immunoassays in agricultural chemistry is localized analysis of pesticide residues. By this we mean quantitative detection of specific residues at sites remote from highly sophisticated, expensively equipped central laboratories. The availability of localized testing will speed data generation, encourage greater volumes of data to be generated, and allow analytical information to be utilized in making crop management decisions. Currently the only comparable practice is soil and plant nutrient analysis in which fertilization programs are influenced by analytical results.

This type of testing can be conducted with simple assay formats which employ no instrumentation or with more sophisticated assays which utilize inexpensive instrumentation. The key features of immunoassays that make this possible are those discussed earlier: assay specificity conferred by the reagent and reactions visualized without radioisotopes or expensive instruments.

Once analytical capability and rapid turn around time are available locally or on the farm, many crop management decisions will be guided by this type of analysis. One example available commercially allows state inspectors to rapidly check that pesticide containers have been properly rinsed before they are discarded. Certain states require a deposit on all restricted pesticide containers. Deposits are refunded only if the container being returned passes a rapid assay which confirms that the container has been properly rinsed. The tests currently used for this on-site analysis are based on immunoassays as well as a colorimetric cholinesterase inhibition assay (26).

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Other on-site immunoassays for chemicals will become increasingly available over the next 5-10 years. These tests will impact a number of economic crop management decisions including:

> Crop rotation patterns based on herbicide levels from previous years treatments. This information would be considered along with data on pathogen and pest levels as discussed earlier.
>
> Choice of chemical treatment based on existing chemical residues, the pest to be controlled, and possible interactions with other chemicals.
>
> Timing of treatment application based upon the rate of loss of active ingredient from earlier treatments.
>
> Harvest scheduling based on residue levels in individual fields. Preharvest intervals are currently based upon "worst case" information from detailed chemical manufacturer trials. In the future, harvest dates may be scheduled by determining whether residue levels in individual fields have reached legal tolerances.
>
> Re-entry period following application of a restricted use pesticide is an area of growing concern. Rapid immunoassays can be employed to determine the dislodgeable residue of specific compounds prior to allowing farm workers to enter a field. On-site immunoassay methods will permit and encourage more active management of work exposure.

Detection and Quantification of Naturally Occurring Compounds. Antibodies can be prepared for naturally occurring compounds as well as for pesticides and drugs. This opens the way for developing rapid immunoassays for plant and microbial products such as mycotoxins, plant hormones, and high value plant components such as flavor and fragrance compounds and pharmaceutical precursors.

Several research groups have reported antibodies for aflatoxins and other mycotoxins (27). Commercial kits for aflatoxin detection in various substrates have been announced. The introduction of such kits will permit on-site detection of aflatoxins to be confirmed immediately rather than having to wait for analytical results from a remote laboratory following detection of fluorescing materials in a commodity. Since aflatoxins and other microbial toxins have a number of structural variations, the antibodies used in their analysis must be carefully selected to assure that the proper compounds are being detected and accurately measured.

Plant hormone analysis tends to be a very difficult task due in part to the low levels that occur naturally. Over the past several years Weiler and his coworkers and a number of other groups have developed extremely sensitive immunoassays for many of the natural plant hormones (28). These assays have greatly reduced the time and effort required to analyze indoleacetic acid, cytokinins, gibberellins, and abscisic acid.

Analysis of Crop Components. The ability of antibodies to bind to a variety of molecules leads to many additional applications in crop agriculture. Once the nature of a target molecule is known, antibodies can be prepared and an appropriate assay procedure devised. For example, antibodies produced to soybean seed proteins are currently being used to detect soybean extenders in meat products. Similar assays could be used directly on soybean seed to select breeding materials which are quantitatively or qualitatively different from existing lines. During production an assay could be used to monitor seed maturation to guide harvest scheduling. Similarly, processors could employ rapid assays for key plant components relating to quality or intended end use of the commodity.

Isoenzyme analysis and electrophoretic separation have been employed to differentiate plant species and varieties but are slow and relatively cumbersome procedures. The background information developed has begun to identify specific markers correlated with desired agronomic characteristics. Using antibodies and immunoassays it is now possible using these rapid tests to identify plants carrying specific genes. Applications of this aspect of agricultural immunoassay apply to the plant breeder who may now screen large populations of seedlings for those individuals which express the specific marker. Further applications include monitoring seed production fields for genetic purity, seed certification, and variety identification at grain elevators and processing plants. As genetic traits are engineered into crops immunoassays can be used to track and certify the new variety. Traits such as specific herbicide resistance are targets for rapid assays since growers may not wish to risk using the herbicide until it is known that the crop is indeed expressing herbicide resistance prior to applying the potentially phytotoxic material.

As basic information in plant science increases additional markers will be identified for which immunoassays can be developed. Rapid determination of the nutrient status of the growing crop will allow crop managers to select fertilization that produces maximum return based on the specific needs of the crop as it grows in a specific location (29). Similarly, detection of water stress markers will permit irrigation scheduling based on crop water status rather than on soil moisture levels or other environmental measures.

Conclusions

Immunoassays are powerful analytical tools that provide sensitivity and specificity comparable to traditional chemical analyses. The advantages of immunoassays lie in their speed, simplicity, and relatively low cost. These factors have fostered the rapid growth of the medical diagnostic industry and have made possible an agricultural diagnostics industry.

Throughout the crop production cycle, managers must make a series of economic decisions about their crops (Figure 5). As the season progresses, each decision represents a greater expense level and, consequently, a greater potential loss. Immunoassay can

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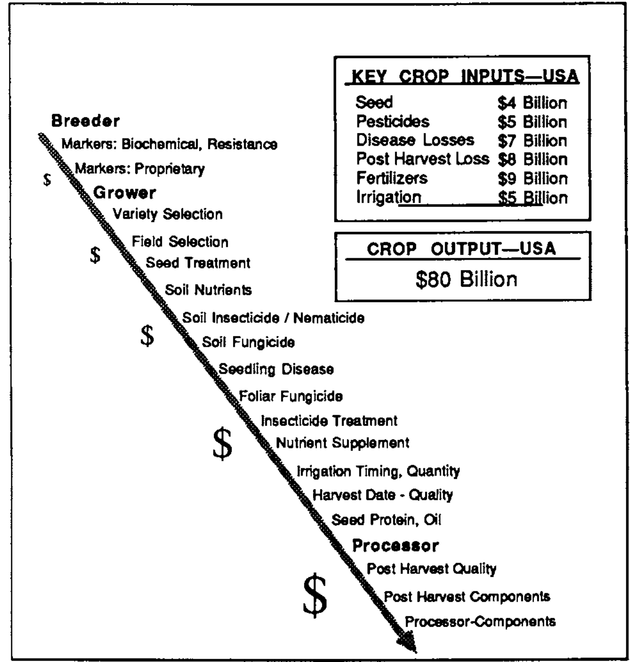

Figure 5. Crop product decisions influence the utilization of variable crop inputs. Crop management decisions occur through all stages of the production cycle. Antibody based assays can provide accurate, timely information to support such decisions.

be applied throughout the crop production cycle to provide specific information. The crop manager uses experience, outside information sources, and careful evaluation of the crop to develop options for managing resources and returns.

The level of farm inputs in the U.S. is very large (Figure 5). As pressure on producers increases, the inefficient will not be successful. The 50,000 growers who are projected to be the source of 75% of America's food and fiber by the Year 2000 will adopt procedures and products to maximize their efficiency. Immunoassays will contribute to that efficiency by providing cost-effective information at the farm, local, regional, and national level.

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Chapter 20

Anticipating the Impacts of Biotechnology on Agriculture: A Review and Synthesis

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The steady increases in productivity improvement that took place in agriculture over the last several decades are beginning to level off, but biotechnologies offer the promise of dramatically shortening the development time for new plant varieties and animal breeds. Cost and risk reductions will be achieved by expanding the potential to install disease resistance, new functions, and expanded production capabilities. The impact of these developments will be felt on the farm level, in competition between regions for commodity production, among the institutions that serve agriculture through research and extension, and in the international arena. This chapter examines processes of technical change in agriculture and the institutional shifts that are accompanying a regional, national, and global restructuring of research and input supply in farming. Consumers ultimately benefit by an expanded supply of cheaper food but uncertainty about the use of new organisms or the effects of a single accident may create problems. Farmers face an accelerated treadmill of technical change that may hasten the trend toward fewer and larger farms. Changes in the U.S. agricultural research system are reviewed as are consequences for Third-World farmers and the international food system.

Biotechnology is affecting agriculture in a number of profound ways. The techniques provide new ways of producing existing substances as well as a new array of chemical tools for improving the efficiency of agricultural production processes. It is becoming increasingly clear that the steady increases in productivity that occurred over the last several decades are beginning to level off as the possibilities of present methods are exhausted and biological barriers are encountered (1). We may be reaching a point of diminishing returns with respect to conventional plant and animal breeding techniques (2).

Biotechnologies present an opportunity to shorten the development time for new varieties and breeds and expand the potential to

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install new functions and capabilities (3-5). A major objective of biotechnology research in plants is to change the genetic makeup of crops to give them increased resistance to environmental and biological stresses (e.g., heat, drought, nutrient deficiencies, insects, diseases) so there will be less reliance on amelioration of these stresses by cultural means (6-7).

The impacts of these developments are being felt at the farm level (8) and in the competition between regions for producing various commodities (9). At the national level, institutional competition for research and development roles in the coming technological revolution is reflected in proposals for revisions in formula funding for the state agricultural experiment stations and the establishment of a National Institute of Agriculture (10-11). At the global level, biotechnology is fueling international competition for primacy in the marketing of new technology and the production of food and fiber (12-13).

One estimate of the rate of technical change in agriculture is that since 1930, productivity has doubled every 30.4 years (corn yields). This rate of change may be compared to computers where technology has doubled productivity every 1.1 years, or to jet aircraft technology (13 years) (14). Biotechnology offers the prospect of accelerating the rate of productivity growth in agriculture. Such increases are vital in the face of exponential world population growth and the need for a corresponding level of growth in the world food supply (15).

Primarily a task of review and synthesis, this paper treats a series of loci of change, focusing on farm-level and consumer consequences. The purpose of this review is to examine the nature and sources of biotechnology's social impacts on agriculture. By definition this is a somewhat speculative effort, as the outcomes of biotechnology research currently are only beginning to be available for use at the farm level (16). But the products are coming and they will affect the economies, industrial organization, and institutional support structures serving agriculture in a number of predictable as well as heretofore unexperienced ways (17-13). We can rely on existing theories and empirical research on technical change in agriculture to explain much that will happen on the farm level (18). But we also must examine institutional shifts that are accompanying a regional, national, and global restructuring of agricultural research and input supply in farming.

What can consumers expect?

For consumers, biotechnology may someday do for food what the microchip did for transistor radios (19). Some foods may be produced in industrial factory settings, bypassing the farm altogether. Yogurt, a single-celled protein (SCP), is a currently available example of a popular non-agricultural food product. New food products based on SCP technologies will compete with farm-based products for the consumer dollar. Lower prices, a new variety of products, and standardized quality may alter the market position of many traditional farm products. In general it seems that biotechnology will be a major basis for productivity increases in the future, expanding supply and keeping prices low, given the relatively inelastic demand for food.

The biotechnology source of new food products will likely be invisible to most consumers. Consumer acceptance of such products will center on basic issues of taste and substitutability for existing products. If conventional pesticides can be supplanted through biotechnology innovations, it may be a means for alleviated consumer concerns about food quality.

Health and safety issues connected to the widespread use of herbicides, and pesticides may alter public perceptions of the relative quality of farm goods versus industrially-produced substances. Volatile issues, such as the effects of new organisms, the untoward consequences of a single accident, and the relative price structure all will enter into the consumer's equation.

What do theories of technical change suggest?

Many of the new biotechnologies will be of great benefit to the farm operator because their lower cost and perhaps improved performance will displace competing products produced by traditional methods (20). Biotechnology products will compete in three major ways: same functions, lower price; improved functions, higher price; and new or additional functions with at least initially premium prices.

Natural diffusion processes are shaped by marketing, product availability, and price (21-22). Given the infrastructure to make the products available and support their initial application and use, farmers adopt new technology in relatively well-understood ways. The supply of an innovation, in terms of distribution in areas where farm operators can readily obtain it, is a primary consideration in the adoption process. If not available, an innovation cannot be adopted. New biotechnology products are likely to be distributed where potential customers are concentrated. In more remote or secondary production regions, lack of product availability may slow the diffusion process.

On the demand side, adoption theory suggests the kinds of operators most likely to implement technology (23). Older, less-educated operators of smaller-scale farms are generally the last to employ new technology and thus receive fewer relative benefits (24). Younger, better-educated operators of larger operations generally tend to use new ideas first, capturing higher returns for their investment over a longer period of time (8).

The nature of the new products may further widen the gap between small and large-scale operations. Biotechnologies may impose significant changes in farm practices if they require expanded understanding of biological processes or otherwise increase the costs of information acquisition (25). The gap between those possessing the expertise (or with the resources to hire knowledgeable assistance) and those relying on their own limited personal resources will be widened. In this sense, biotechnologies may accelerate current trends toward a bimodal distribution of many small and more large farms.

How will biotechnology affect farmers?

Impacts on agricultural producers can be considered in two major

ways: on farmers as a collective whole, and on subsets or classes of farm operators. One major consequence for farmers as a group is associated with the pricing of innovations and the capturing of benefits from improved productivity. Who benefits: farmers, middlemen, or the biotechnology companies? For many crops, hybrids ensure repeat business for successful products because growers are unable to replicate the parent crop characteristics (26). Sophisticated pricing ensures that adoption incentives are sufficient to propel widespread diffusion but that the lion's share of marginal productivity goes upstream and not to the producer.

A related consequence of widespread adoption of a supply-increasing biotechnology product is lower prices for the farm product, as demand for most farm commodities is relatively inelastic. A forthcoming bovine growth hormone is projected to increase milk production 10 percent (27). Falling prices will press less efficient producers to alter their operations or go out of business. As few individuals will be able to enter the business, the industry will shrink and concentrate production in fewer firms.

Some classes of farms will be differentially affected by new products that provide a quantum (rather than incremental) jump in productivity or efficiency. The firms most likely to be profitable are those that can best incur the risk, those that have the highest performance aspirations, and those with access to the most information about the innovation (28-29). Thus larger farms seem to have an advantage with respect to the advent of new technologies.

The optimum size for the most competitive farm firm has been the subject of much research (30). Past studies of the relationship between average production costs and farm size reach two major conclusions. First, most economies of size are apparently captured by moderate-sized farms. Second, while the lowest average cost of production may be attainable on a moderate farm, average cost tends to remain relatively constant over a wide range of farm sizes. Thus, operators have a strong incentive to expand the sizes of their farms to increase total profits. In addition, external economies gained from buying and selling in large volumes, from access to credit, government subsidies and tax advantages may further lower average production costs for larger units.

In assessing the advantage to size, it is important to consider complementary inputs that might be used with new biotechnology products. Consideration must be given to entire technology packages, not just the isolated new biotechnology input. This notion has received substantial attention in evaluating size effects of technology in an international context (31-32). In addition, U.S. small-farm operators are quite heterogeneous with respect to age, off-farm employment, and orientation to farming making generalizations related to size effects difficult.

What are the regional impacts?

The potential exists for significant regional changes in location of agricultural production as a result of new agricultural biotechnologies. Preliminary results from one economic model of the farm sector suggest, in the case of the bovine growth hormone (bGH) used in dairy cattle, that dairy-importing states may be the major bene-

factors and exporters the major losers (9). Biotechnology products may be able to overcome some of the regional disadvantages related to physical production characteristics, i.e. climate and soil. Reduction in regional comparative advantage may make producers in physically disadvantaged areas the net gainers and the losers may be areas currently possessing comparative physical advantages.

The areas of comparative physical disadvantages will reap these benefits only if agricultural experiment stations are successful in adapting technology to local conditions and extension services are successful in convincing farmers to adopt early. It is possible to envision significant changes in terms of trade among various production regions as a result of new biotechnologies. The location of agricultural production may be based more on transportation cost and other marketing considerations and less on production considerations such as soil fertility and climate.

How are input and service industries affected?

Biotechnology is altering the structure of existing industries and creating new industries as the discoveries unfold. Perhaps the most dramatic example of restructuring is the seed industry, particularly the production of hybrid corn (33-34).

Seed from open-pollinated varieties of corn can be saved for replanting, but hybrid seed tends to revert to the character of the original inbred lines after the initial expression of optimum traits in the hybrid generation. The hybrid is proprietary in character as the particular inbred lines can be kept secret. The farmer is motivated to use the proprietary hybrid over purebred varieties because of the relative advantages of the hybrid in terms of such qualities as superior yield and increased disease resistance. Kloppenburg (33) argues that these characteristics provide seed companies with the opportunity to break down the autonomy of the farmer, retain the gains to proprietary research, and enlarge profit margins.

Since the 1930's, the rapid adoption of hybrid seed corn has fueled the development of the seed industry. Hybrid sales rose on the same trajectory as corn yields to around a billion dollars today. Because the offspring of hybrid seed are not reliable, farmers were guaranteed to be repeat customers and profits flowed steadily to the seed industry. As Kloppenburg (33) notes, the sustained profitability of seed companies had brought most of the seed industry into multinational petrochemical and pharmaceutical corporations. Only Pioneer-Hybrid remains an independent firm.

The experience of hybrid corn has set a precedent and suggests a strategy for private sector control of the seed business. Some crops are dominated by hybrids while others are not. Hanway (35) estimates that 85 percent of the corn varieties used by farmers in 1977 were privately developed, but only 3 percent of the peanut varieties were. About 89 percent of soybean varieties were publicly developed. Only 5 percent of the corn seed market is supplied by farmers for home use or from local sale; but more than 60 percent of soybean, wheat, and oats are from these independent sources (33). Other untoward effects of this shift relate to over-dependence on a few varieties that may increase the vulnerability of the nation's corn crop to disesase through overly focused genetic backgrounds.

Some feel that the Plant Variety Protection Act is one step to ensuring farmer dependence on external sources of seed supply because it institutionalizes breeders' marketplace rights (36). As a result of a recent decision by an appeals board of the U.S. Patent and Trademark Office, genetically engineering plants, seeds, and tissue cultures can now be patented. Previously, plant breeders could obtain protection for single varieties even though the modification could be installed in many others. Now a patent can be issued genericly to all varieties with the change.

Industrial concentration in corn and the other seed industries is likely to be exacerbated by unfolding biotechnical advances. The pharmaceutical and chemical companies possessing the biochemistry research base have acquired the seed companies to deliver the ultimate product of their innovations: the seed. The programming of the genetic code in new varieties will proceed on the basis of profitability, but it is not clear how concerns over environmental sustainability, genetic vulnerability, and distributive justice will factor into these equations.

Concentration in the industry also raises the specter of monopoly control of a nation's seed supply. Monopoly control is a concern for two reasons: the potential for predatory pricing to farmers and ecological vulnerability stemming from overdependence on a narrow set of plant varieties. The discovery of a particularly effective hybrid in a specific crop could lead to widespread adoption. This may threaten national or world supplies if genetic lacunae later contribute to crop failure. The demands of capital in these industries are exerting clear pressure on the public research sector to focus on "upstream" grandparent line development and leave the "downstream" hybrid variety work to the private sector (37).

Although there are exceptions, the complementary relationship of human-related biotechnology research to animal-related research favors the earlier development of products for animals than plants. Basic cell processes are better understood for animals than for plants, and plant characteristics have much more complex genetic mechanisms (38). Private firms already involved in pharmaceutical research can easily move into animal agricultural biotechnologies. Dairy, beef, swine, and poultry will be the leading markets for biotechnology products in the near future. Regulatory considerations also favor the development of animal products because gene splicing and embryo transfer, the dominant scientific techniques used in animal biotechnologies, do not involve release of genetically-engineered materials into the environment. However, health and food safety issues remain as potential hurdles for both animal and plant products (39).

Many previous agricultural chemical agents have had a relatively wide spectrum of efficacy. Vaccines and monoclonal antibodies based on rDNA techniques seem to be very effective against specific agents. But where multiple sources of infection are involved, the specificity of monoclonal antibodies is a disadvantage. Similarly, microscopic organisms have relatively high rates of evolutionary change. Thus the "staying power" of rDNA approaches to disease prevention remains to be fully defined. Farmers may be faced with a bewildering array of specific products further increasing the complexity of production activities.

What kinds of institutional impacts can be expected?

Traditionally, the nation's farm productivity improvements have largely originated in university-based agricultural experiment stations as coordinated by USDA's Cooperative States Research Service. Complemented by the USDA's Agricultural Research Service as well as private sector laboratories, this system has made U.S. agriculture the most productive and efficient in the world (40).

The primary vehicle for accomplishing agricultural research has been the agricultural experiment station system. Much of the work has been characterized as a "downstream" or "technology development" research (37). Basic research has been concentrated at a few larger well-endowed core institutions and the USDA's Agricultural Research Service (41-11). Most of the advances in biotechnology have emanated from these core institutions with long-standing programs in molecular biology.

The advent of biotechnology has brought the segmented structure of agricultural experiment station research into sharp relief. The have's and have-nots (in terms of research capability) have started a scramble to catch up. The public agricultural research system's inefficiencies and lack of attention to basic research is not a new issue (40-5).

A conference held in Winrock, Arkansas sponsored by the Rockefeller Foundation and the consequent Winrock Report marked a watershed in the evolution of the Land-Grant System (42). Some participants felt that the experiment station research system should be dismantled and a National Institute of Agriculture patterned after NIH organized to guide agricultural research (43). This and subsequent proposals to greatly expand competitive funding processes revealed certain cleavages in the agricultural experiment station system. The core set of agricultural experiment stations favored expanded competitive funding. The Midwest tended to favor revising the funding formula, as their states did not proportionately support agricultural research as much as do the Southern states. For the most part these efforts were beaten back, although a small competitive grants program was finally put in place, but only in terms of newly-appropriated monies.

The experience did shake the system (41-44). The Agricultural Research Service was reorganized to focus more attention on basic research. "Biotechnology" began to diffuse to the periphery of the Agricultural Experiment Station system. Pareto's "80-20" rule nearly applies, as about 28 percent of the stations do more than 80 percent of the biotechnology research (45).

Nevertheless, the question of productivity growth in agriculture remains an issue (2). In the present environment, cost-cutting is more important than increasing yield for the farmer (although he would like to do both). Agricultural Experiment Station technology transferred through Cooperative Extension Service county agents has been the accepted model for introducing these improvements. Today, farmers are tending to directly utilize experiment station research and to rely on the county agent as more of a coordinator for local activities serving agriculture. Technology often is directly conveyed to the farmer by private industry though input suppliers, farm magazines, and other media (46-47).

For several reasons, biotechnology products are more likely to flow through the market conduit than through public institutions. Patent protection may be less useful for biological innovations, even with the Supreme Court ruling that genetically altered materials should be considered different materials, thereby patentable. Proprietary control of biotechnological products will be much more problematic. The presence of an active ingredient in an agricultural chemical can be readily identified, and patent rights can be enforced. The uniqueness of particular alterations of genetic material will be more difficult to identify and it will be more difficult to control the abuse of proprietary rights. the private sector has responded by wanting to regiment the process from the very beginning and imposing tight restrictions on any information concerning new product development. The shift toward more private sector involvement in basic research results from the assumption that patent rights will be difficult to enforce.

Butler and Schmid (17) contend that much of the work that goes into cosmetic breeding in meeting patentability requirements is wasted effort since it is not possible to define under current patent laws "how different is different." They point out that the solution is not simple. On the one hand, if regulations are changed to create more specific guidelines for patentability resulting in a narrow product-space, then we allow for the possibility of substitutes but there may be fewer incentives for innovative research. On the other hand, if patent regulation allows patents to be taken out on the basis of broad product-space definitions, then we end up with a monopoly and no substitutes. It is not clear whether institutional procedures exist that will allow exclusive rights without monopoly and still create incentives for innovative research and the release of competitive substitutes (13).

What are the safety and regulatory issues?

One fear is associated with agricultural biotechnology is the potential virulence of altered organisms, or the ability of new organisms to gain a selective advantage. The USDA section of a more recent Office of Science and Technology Policy (OSTP) document (48) does state that, to date, no unique or safety problems have been associated with products of genetic engineering, conventional or modern. One can point to the African "killer bees" in California or the rapid-growing Kudzu vine in the South as examples of conventional scientific research gone awry. It is not unreasonable to expect that similar unfortunate mistakes or accidents will occur in the future.

For decades nonindigenous organisms have been introduced into the U.S. that contributed greatly to our food sources and provided new ornamental species. Agricultural scientists have been able to create new gene combinations in single organisms -- even new species -- through mutagenesis, cross-hybridization, and other breeding techniques (48). The argument for confidence in biotechnology safety is that these products are not fundamentally different from products obtained from conventional technology (39). On the other hand, we have never experienced the accelerated power to do these and other manipulations, nor has the enhanced capability been so widespread around the world.

On the last day of 1984, the Office of Science and Technology Policy (OSTP) issued a proposal for regulation of biotechnology in the Federal Register. This document provides a concise index of U.S. laws related to biotechnology, identifying the major regulatory agencies that review research and product development. It is an effort to coordinate the Federal role in the assessment of biotechnology as it moves from contained research laboratories into full contact with the public and the environment through commercial development and actual application (48).

Two major perspectives seem to be emerging. One argues that biotechnology products present marginal or incremental improvements to organisms, that we already have no trouble living with, that the survivability of altered cells in the environment is very low, and that few if any practices beyond those already implemented are necessary. Alexander (49) maintains that the probability of a deleterious effect from a genetically engineered organism is a product of six factors: release, survival, multiplication, dissemination, transfer, and harm. The odds of simultaneous occurrence of all these conditions is low. The concern here is that regulatory overreaction and inefficiencies will stifle innovations and hurt the competitiveness of U.S. industry.

Large-scale release biotechnologies seem to be the most worrisome and controversial. These involve general release of a genetically engineered microorganism into the environment and thus have a high potential for imposing costs on others (50). Some bacteria that colonize crops, for example, serve as a nucleus for the formation of ice crystals thus making crops more vulnerable to low temperature. A proposed experiment to introduce bacteria stripped of the ice nucleus forming gene into farm fields was stopped by the courts. It was ruled a full environmental impact statement was required. Those opposing the ice-minus bacteria experiment argue that we simply know too little about the consequences of allowing novel organisms to be introduced willy-nilly into the environment. Assessing the risks of large-scale release technologies will be difficult as standard testing protocols have not yet been developed.

Most scenarios mentioned by scientists for costs emanating from biotechnology applications are accidents that result from unintended incursions into the ecological system (51). Biotechnology may create interactions between systems not previously linked, and perhaps could not be foreseen to be linked. Perrow cites the unexpected effects of DDT and related pesticides as a precedent for concern. The danger that Rachel Carson (52) publicized was not direct poisoning (which is observable and well-understood), but of the magnification of the substances in living tissues as they move up the food chain. Thus, the catastropic or harmful potential of biotechnology lies in unintended interventions into the ecosystem.

Experiments show that rDNA research could create a vehicle for the transmission of hazardous traits (51). Although of low probability, the idea is that the peculiar and subtle complexities of recombinant organisms might lead to serious health hazards simply by interacting with biological systems in new and unanticipated ways. The pressures of economic competition for patents and commercial applications would seem to exacerbate these risks (53). Although

biotechnology may be a source of a cure for disease (54), it may produce successor scourges through human accident. Parallel threats to domesticated plants and animals can be envisioned.

What is happening in international agriculture?

Biotechnology offers a high potential for improving the world's food supply. It also presents the possibility of expanded corporate control over Third World economies and a diminished role for some food-producing countries in the world market place (55).

As discussed earlier in the section on industry impacts, hybrid plant varieties shift marketing control upstream to the seed companies and away from the farmer. Linkages between plant varieties and certain proprietary inputs may constrain farmers to technology packages that increase farmer dependence on sole-source input suppliers. Although Third World farmers may produce more food with biotechnology products, part of the profits will be shipped to the developed-country homes of multi-national corporations. The question is over the relative share received by the farmers, the developing countries, and the multinational corporations.

Buttel (43) identifies a series of concerns for international agriculture. 1) Will the concentrated agricultural industries extract monopoly rents from Third World producers? 2) Will the biotechnology that is developed and marketed increasingly constrain the farmer to "biotechnology packages" that lock-in the market for proprietary inputs as well as the seed (such as atrazine-resistant corn). 3) Will proprietary concerns constrict the flow of scientific information to public sector researchers responsible for generating technology for small farmers and smallholding peasants? 4) Will biotechnology produce "one-shot" products of such comparative advantage that multinational seed companies will dominate Third World markets?

Biological innovations tend to be associated with increases in output per land area (56); they are generally land-saving, not labor-saving. Biotechnology products will be favored where land prices are relatively high. In an international context, agricultural producers in land-short Japan and Taiwan, and perhaps some of the European countries will be most likely inclined to implement these technologies.

Buttel (11) fears that past experience leads us to expect increased socioeconomic differentiation, increased reliance on capital-intensive production, much greater off-farm than on-farm benefits to small farmers. The bright side may lie in an expanded supply response which may allow food production to keep pace with the exponential growth in population although the immediate problems of world hunger involve distributional problems rather than inadequate supply.

Conclusion

Technology assessment (TA) is a broad-scope effort to examine the potential consequences of a new technology (57). Technology asessment deals comprehensively with a full range of social, economic and environmental impacts. It is oriented toward policy decisions by a legislature, government agency, or, occasionally an industrial cor-

poration. Technology assessment is an attempt to anticipate consequences beforehand rather than waiting for them to become evident. The approach endeavors to identify a wide range of impacts, beneficial as well as adverse, social and political, environmental and economic. Although government and industry often are central actors, there are people who face significant indirect costs who have no effective voice. Thus, technology assessment requires the identification of the main parties to be affected and an analysis of the consequences for each party.

This paper has briefly assessed some of the impacts of biotechnology in agriculture. This is a difficult if not impossible task because biotechnology, even if restricted to agricultural issues, is an aggregate of diverse techniques, tools, and applications. So many developments are occurring on so many fronts that conclusions are difficult to draw. We have, however, attempted to identify some of the generic processes of technical change in agriculture as a framework for understanding the lifecycle of innovations, their evolution, and spread in the industry.

It is also possible to oversell the effects of biotechnology on agriculture. It is reasonably clear that technology is one of the driving forces behind structural change in agriculture (30). Yet, continued improvements in mechanization, information technology, as well as other biological advances will contribute to increases in agricultural productivity. The direction and rate of technological change will be shaped by shifts in the relative prices of production inputs, government policies, and conditions in the world marketing system. Disentangling the independent effects of each locus of change will be a complex undertaking (58). Nevertheless, biotechnical developments represent a significant component in the overall equation.

Jacques Ellul (59) maintains that technical progress is always ambiguous, it is neither good nor bad, the contradictory elements are indissolubly connected. Four of his main ideas are: 1) all technical progress exacts a price; 2) technique raises more problems than it solves; 3) pernicious effects are inseparable from favorable effects; and 4) every technique implies unforseeable effects. Thus biotechnology is not likely to solve many agricultural problems and will undoubtedly create a host of new ones.

Future efforts to understand the panoply of changes emanating from biotechnology must focus on more delimited subsectors of agriculture (plants or animals), particular technologies (fermentation, rDNA, etc.), and specific applications (bovine growth hormone, ice-minus bacteria, etc.). Using existing theories and past experiences the evolution of particular technologies, their industry impacts, farm-level implications, and societal consequences can be better understood. From this understanding perhaps benefits will be more widespread, adverse affects avoided, and wiser decisions made.

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Chapter 21

Patent Laws Governing Applications of Biotechnology Patents Related to Agricultural Products

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The Supreme Court Decision in Diamond v. Chakrabarty, 477 U.S. 303, 206 USPQ 193 (1980) paved the way for patenting living microorganisms and described Section 101 of the general patent laws as encompassing a broad range of subject matter including plants. Plants can also be protected by the Plant Patent Act of 1930 and the Plant Variety Protection Act of 1970. However, both of these acts contain special provisions and limitations not found in the general patent law. Questions have arisen whether plants protectable by one of the latter acts can also be protected under the general patent law. This issue was the subject of a test case which arose in the Patent and Trademark Office. Special considerations and questions arise in obtaining patent protection for agricultural biotechnology patents. Examples of issues include the scope of patent protection, utility, disclosure and deposit requirements, and claim techniques. Patent Protection, especially for biotechnology, is being strengthened in the United States and efforts are being made to strengthen intellectual property protection in foreign countries.

Plants, methods and apparatus for breeding or modifying plants and their characteristics and other plant-related and agricultural products and methods can be protected by trade secret or by patent or a patent type of protection. Patents can protect large investments in research and development against competitors who copy proven-successful products without the same investments. Patents are available for processes including genetic engineering processes and techniques and products including DNA segments, plasmids, vectors, hybridomas, transformed cells, pure cultures, monoclonal antibodies, proteins, etc. as long as they are made by or are the result of human intervention. Such processes and products, of course, must meet the other statutory criteria

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for patentability; they must be "new and unobvious" processes or products.

Trade secret protection has the advantage that any subject matter which can be maintained in confidence may be covered by trade secret protection for as long as its confidentiality is preserved. However, trade secret protection is lost when the subject matter is no longer confidential. If it can be reverse-engineered or if others develop the same technology or legitimately learn of it, protection is lost. It may even be lost to the public where information is initially obtained illegally and made public. While patent-type protection can only be obtained for new and unobvious subject matter and disclosure is required, there is absolute protection for a fixed term against copying and later independent development. A patent permits the owner to exclude others from making, using and selling the subject matter covered by a patent for a period of 17 years.

Let's review four policy or issue areas in the patenting of plants and related plant and agriculture methods and products: (1) Patenting Plants Under General Patent Law, (2) Extension of the Term of Agricultural Patents for Regulatory Clearance Delays, (3) Strengthening Patent and Intellectual Property Protection Especially Biotechnology in the United States and Internationally, and (4) Specific Patenting Issues.

Patenting Plants Under the General Patent Law

Three separate laws provide patent-type protection for plant life itself, depending upon the reproductive nature of the plant.

Sexually reproduced plants, except fungi, bacteria and first generation hybrids are protected by the 1970 Plant Variety Protection Act or PVPA. This Act provides for the issuance of a certificate of plant variety protection from the Plant Variety Protection Office of the Department of Agriculture which administers the Act. The protection granted provides protection against unauthorized propagation of seed to be sold or used as a propagating material for a period of 18 years.

Asexually reproduced varieties including cultivated sports, mutants, hybrids and newly found seedlings, other than a tuber propagated plant or plant found in an uncultivated state, that is, according to the legislative history, the Irish potato and Jerusalem artichoke varieties, may be protected under the 1930 Plant Patent Act or PPA by a plant patent issued by the Patent and Trademark Office. By judicial interpretation, bacteria are also precluded from protection under the Plant Patent Act. Protection for a 17 year term is provided against the unauthorized asexual reproduction of the plant, or use or sale of the plant.

Living plants or other subject matter were considered not patentable uner the general patent law until recently. In accordance with the 1980 decision of the Supreme Court in Diamond v. Chakrabarty 447 U.S. 303 (1980), the general patent law does not exclude the patenting of man-made living matter. In deciding if an invention is a "manufacture" or "composition

of matter," and hence patentable subject matter under the general patent law, the fact that the subject matter may be living made no difference to the Court. The Court gave wide scope to section 101 of the general patent law indicating that the relevant distinction between patentable and non-patentable subject matter was not whether the subject matter was living or not, but whether the subject matter was merely a product of nature or the result of human intervention. The Court indicated that the PPA and PVPA were not enacted to permit the patenting of living things but to avoid the product of nature problem and the strict disclosure requirements of the general patent law. The Court's discussion of section 101 of our patent law, and the purposes and effects of the Plant Patent Law and the Plant Variety Protection Act, makes clear that plants are within the subject matter that can be patented under section 101 of the general patent law. The Chakrabarty decision, however, did not explain or define the jurisdictions over plants (whether sexually or asexually reproduced) of each of the three laws.

The provisions of each of these three laws, the Plant Variety Protection Act, the Plant Patent Act and the general patent law under section 101 of title 35 U.S.C., differ in a number of important respects. The requirements for an application differ; the processing of applications and administration of the laws differ; and the scope of or limitations on the exclusive rights granted by each differ. For instance, under the PPA, the description need only by as complete as is reasonably possible; however, only a single claim to the whole plant, not its fruit or flower, may be presented; and the right to exclude is limited to sexual reproduction of the plant or selling or using a plant so reproduced. Under the PVPA, deposit is required, the criteria for "patentability" or protection differ and there are certain limitations as to what constitutes infringement including a compulsory licensing-type provision and an exemption for farmer's crops and for experimental use.

Until recently, plants, as living subject matter, were not considered patentable under the general patent law. However, as already indicated, the Supreme Court in Diamond v. Chakrabarty, decided that a living genetically engineered microorganism was patentable subject matter under section 101 of title 35.

Since the Chakrabarty decision, the Patent and Trademark Office has adopted the interpretation of the three laws governing plants that any subject matter protectable under either the PPA or PVPA cannot be protected under the general patent law (35 U.S.C. 101). This interpretation is based on application of certain principles of statutory construction.

In light of Chakrabarty, it appears that section 101 specifically protects man-made life forms at the microorganism and plant level, including plant life itself. However, in the 1930 Plant Patent Act and the 1970 Plant Variety Protection Act, Congress has specifically set forth how and under what conditions plant life covered by these Acts should be protected. Since the plant patent provision was added to the predecessor of section 101, it is clear that Congress intended a "distinct

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and new variety of plant" covered by the Plant Patent Act to be something apart from the statutory categories of invention embraced by section 101. Also, the Plant Variety Protection Act of 1970 was enacted to provide a specific form of protection for the breeders, developers, and discoverers of novel varieties of sexually reproduced plants, as opposed to the asexually reproduced plants covered by the Plant Patent Act. Section 41(a) of Title 7 defines novel varieties to include "seed, transplants, and plants." Congress could have amended section 161 of Title 35 to remove the asexual reproduction limitation, but elected to provide separate protection with different provisions to be administered by the U.S. Department of Agriculture.

Where separate statutes dealing with general (35 U.S.C. §101) and specific aspects (PPA, PVPA) of the same subject appear to conflict, the statute specific to the subject should not be controlled or nullified by the general one in the absence of specific Congressional intent. Bulova Watch Co. v. U.S. 365 U.S. 753 (1961); Morton v. Mancari, 417 U.S. 535 (1973); Sutherland Stat. Const. Section 51.05 (4th Ed.). Unless a later statute is irreconcilable with an earlier one dealing with the same subject matter, courts are loathe to find repeal of the earlier statute by implication. United States v. Greathouse, 166 U.S. 601, 605 (1896); Washington v. Miller, 235 U.S. 432, 428 (1914).

Both the Plant Patent Act (PPA) and PVPA contain many provisions or limitations that are unique to plant subject matter and which are not consistent with protection granted to 35 U.S.C.101 subject matter. There is nothing in the Supreme Court's decision in Diamond v. Chakrabarty, 447 U.S. 303 (1980), which was not concerned with plant life per se, that could be interpreted to sweep away features which are unique to protection offered under PPA or PVPA and which constitute an explicit expression of Congressional intent to provide specific protection to plant life covered by these Acts. For example, (1) the PVPA contains both research (experimental use) and farmer's crop exemptions - it is not clear that either would exist with section 101 protection; (2) PVPA spells out infringement in great detail and includes a compulsory licensing provision - no such Congressional guidance exists under section 101 protection; (3) the PVPA limits protection to a single variety, whereas the opportunity for greater and broader exclusionary rights exists under section 101 protection; (4) under 35 U.S.C. 162, applicant is limited to one claim in formal terms to the plant shown and described; and (5) under 35 U. S.C. 163, the plant patent grant conveys the right to exclude others from asexually reproducing the plant or selling or using the plant so reproduced. It would thwart the apparent intent of Congress if plants protectable under the PPA or PVPA with certain specific limitations, were also protectable under Section 101 where such specific limitations do not apply.

The Patent and Trademark Office presently has a test case before it with the issue whether subject matter protectable under the PVPA can be protected under the general patent law.

The Office has attempted to speed the processing of the application involved as much as possible so that this statutory construction issue is resolved as soon as possible at the Board of Appeals and Interferences level in the Patent and Trademark Office and, if necessary, at the Court of Appeals for the Federal Circuit or the district court. The application involved was given a first action rejection in January, a final rejection in March and was recently appealed to the Board of Appeals and Interferences. A hearing was held before the Board on August 9, 1985 and we expect a decision from the Board any day. (Subsequent to this presentation on September 18, 1985, the Board of Appeals and Interferences, in Ex Parte Hibberd (227 USPQ 443), decided this test case holding that plants protectable under the PVPA are also protectable subject matter under section 101 of the general patent law. The Board found no irreconcilable conflict between PVPA provisions and the general patent law.)

This issue is important to whether or not a patent owner can get the strongest possible protection for a plant. A number of advantages are associated with patent protection under the general patent law over the PPA and PVPA. Broader subject matter can be claimed and protected under the general patent law. A whole species of plant may be able to be claimed and protected under the general patent law rather than only a specific plant as in the case of the plant laws. A simplilfied claim format may be used where a broad species is claimed in so-called "markush" claims. A "doctrine of equivalents" clearly applies giving broader scope to claims under the general patent law. On the other hand, disclosure requirements are certainly more rigid than under the PPA and a deposit of the plant in a public depository may be required as in the case of the PVPA.

Other issues of patentability under section 101 of plant tissue cultures and plant parts such as cuttings, fruits and flowers, gene or gene segments of plants, synthetically produced seeds, etc. also exist.

Patent Term Extension for Agricultural Products

The Drug Price Competition and Patent Term Restoration Act of 1984 (P.L. 98-417), enacted on September 24, 1984 provides for the extension of the term of a patent for up to five years where marketing of the product covered was delayed because the product is subject to a regulatory review period before the Food and Drug Administration. Products covered include human drug products, medical devices and food or color additives. The Act recognizes that the effective patent term may be cut short of the normal 17 years unfairly because of long delays in the regulatory clearance process.

This Act did not apply to agricultural products where similar delays caused by pre-marketing clearances exist. Other bills which would cover agricultural products in the last Congress did not pass. However, legislation is pending in the current Congress which would permit the extension of the term of agricultural products in a similar manner. S.1093 would provide for extensions of the term of a patent up to five years for

agricultural and industrial chemicals and animal drugs where pre-marketing clearances are required for such products from the Food and Drug Administration, the Department of Agriculture or the Environmental Protection Agency. A hearing was held by the Patent, Copyright, Trademark Subcommittee of the Senate Judiciary Committee on S.1093 on September 10, 1985. The Administration supports the objective of this legislation.

<u>Strengthening U.S. and Intenational Property Protection for Biotechnology</u>

Special focus has been placed on strong, quick patent protection for biotechnology, especially for genetic engineering technology including cloning, gene transfer and somatic cell genetics. Technology is literally exploding in this area and patent protection is of great importance. As a result, the Patent and Trademark Office has taken steps to:

(1) consolidate most biotechnology including genetically engineered plants, but not conventionally developed plants, in one of our patent examining groups, Group 120, to assure more uniformity in the handling of patent and technical issues.
(2) consolidate, reorganize and beef-up our search files for genetic engineering,
(3) increase our staff significantly to handle the mushrooming applications for biotechnology,
(4) broaden the expertise in our staff to cover the various disciplines involved in this technology including molecular biology, physical chemistry, etc. as well as plant breeding and pathology,
(5) strengthen internal training programs in the technology, and
(6) create a dialogue with the patent bar regarding legal issues in patenting in this field.

A Cabinet Council Working Group has been created and is reviewing the current state of industrial property protection available for biotechnology and is reviewing recent recommendations made by an OECD group of experts to strengthen the protection of biotechnology around the world. A U.S.-European Community Working Group on High Technology has met to discuss intellectual property rights for biotechnology (as well as for computer software and chips), also with focus on the OECD biotechnology recommendations.

The OECD expert recommendations included creation of an international grace period within which a patent application can be filed after publication of the technology, provision for claiming microorganisms in patents, including naturally occurring microorganisms which are isolated and identified, more common provisions on deposits of microorganisms and when they must be released for public access, stronger plant protection, and other provisions relating to streamlining the patenting and enforcement processes and encouraging positive

and clear patent policies by universities and research organizations and scientists. The United States already has in effect provisions and policies in line with the OECD recommendations. But many other countries do not. The World Intellectual Property Organization (WIPO), a United Nations specialized agency, has convened a Committee of Experts which has also initiated a study of biotechnology protection needs. A consultant's report was completed and published in July. Further, a separate group under WIPO and affiliated with the International Union for the Protection of New Varieties of Plants (UPOV), is studying plant breeders rights protection expecially for genetically engineered plants.

In addition to the patent term extension legislation, the last Congress passed a large number of bills to strengthen intellectual property protection in the United States and internationally. Many of these bills at least indirectly strengthen intellectual property for plants and agricultural products and processes. For instance, amendments to the Patent Law eliminate most of the so called secret prior art, that is information known only within an organization or company and not publically, so that it cannot defeat patentability as was often the case previously. Amendments to the Tariff and Trade Act provide that application of the Generalized System of Preferences (GSP) to other countries must take into consideration the adequacy of the countries intellectual property protection for U.S. interests. Also changes were made to reduce the antitrust risks of entering joint ventures by limiting damages for antitrust violations in joint ventures noticed to the Department of Justice and the Federal Trade Commission. Other legislations is pending in addition to patent term extension legislation for agriculturally-related products including extending process patent protection to products made by the protected process and sold in or imported into the United States.

Examining and Infringement Issues for Genetically Altered or Engineered Plants and Agricultural Products

A variety of issues exist relating to applications for genetic engineering products and processes. These are generally similar to issues that have come up for many years in regard to chemical products and microorganisms. These issues relate to deposits, disclosure, claim drafting and infringement. The analogy to existing chemical practice is good and helps resolve many of the issues. However, some of the existing concepts must be applied to some new fact situations that are not always perfectly or clearly analogous.

Description Requirement.

Section 112 of Title 35 requires for applications filed under the general patent law that the specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise

and exact terms as to enable any person skilled in the art to which it pertains or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The function of the description requirement is to ensure that applicants had in their possession, as of the filing date of the application relied on, the specifc subject matter claimed by them. In re Wertheim 541 F2d 257, 191 U.S.P.Q. 90 (CCPA 1976). It is not necessary that the application describe the claim limitations exactly, but only so clearly that persons of ordinary skill in the art to which the invention pertains would recognize from the disclosure that applicants' invention included those limitations. In re Smythe, 480 F2d 1376, 178 U.S.P.Q. 179 (CCPA 1973). The written description consists of the specification and the claims as orgiinally filed. In re Gardner, 480 F2d 879, 178 U.S.P.Q. 149 (CCPA 1973). Where the invention relates to living subject matter, unless the invention can be adequately described by words alone or other descriptive information, a deposit may be required to satisfy this requirement.

When a description requirement issue arises, the PTO has the initial burden of presenting evidence or reasons why persons skilled in the art would not recognize in the disclosure a description of the invention defined by the claims. For example, the PTO's burden of proof would be satisfied when the examiner points out a claimed embodiment (e.g., added by amendment) outside the scope of the disclosure as filed. The burden of proof then shifts to applicants to show that the claimed invention is part of the description as filed.

Enablement.

The essence of the enablement requirement is whether the disclosure contains sufficient teaching regarding the subject matter of the claims as to enable one skilled in the pertinent art to make and use the claimed invention without undue experimentation. The critical question is whether the scope of enablement provided to one skilled in the art by the disclosure is commensurate with the scope of protection sought by the claims. In re Moore, 439 F2d 1232, 169 U.S.P.Q. 236 (CCPA 1971).

Currently one of the most prevalent standards for measuring sufficient enablement to meet the requirements of the first paragraph of 35 U.S.C. 112 is that of undue experimentation. The test is whether there is sufficient working procedure for one skilled in the art to practice the claimed invention without undue experimentation. In re Stephens, 529 F2d 1343, 188 U.S.P.Q. 659 (CCPA 1976). The test of enablement is not whether any experimentation is necessary, but whether, if experimentation is necessary, is it undue. The experimentation required also must not require ingenuity beyond that expected of one of ordinary skill in the art. In re Angstadt, 537 F2d 498, 190 U.S.P.Q. 214 (CCPA 1976). Specific factors which are considered in determining whether or not experimentation required is undue are (1) presence or absence of a working example, (2) nature of the invention, (3) state of the prior art, (4) relative

skill of those in the art, (5) the predictability and unpredictability of the art, (6) the breadth of the claims and (7) the quantity of experimentation necessary. In re Collanni, 561 F2d 220, 195 U.S.P.Q. 150 at 153 (CCPA 1977).

Deposits of microorganisms or like material are not a requirement of the statute, but are one way an applicant may choose to satisfy the requirements of 35 U.S.C. 112. In re Argoudelis, 434 F2d 1390, 168 U.S.P.Q. 99 (CCPA 1970). Feldman v. Aunstrup, 517 F2d 1351, 186 U.S.P.Q. 108 (CCPA 1975). Deposits are not necessary to provide enablement where the invention relies on known, publically available organisms and can be practiced as described with only routine experimentation. Tabuchi v. Nuabel, 559 F2d 1183, 194 U.S.P.Q. 521 (CCPA 1977).

In many biotechnology-related inventions the issue is often raised as to whether to deposit the starting materials and/or final product to satisfy the requirements of the first paragraph of 35 U.S.C. 112. This issue can only be resolved by considering the availability of the materials used and claimed and the reproducibility of the techniques utilized to obtain the desired product from the starting material (if not otherwise publically available).

Enablement is judged as of the effective filing date of the patent application being considered. White Consolidated v. Vega, 713 F2d 788, 218 U.S.P.Q. 961 (Fed. Cir. 1981). In re Glass, 492 F2d 1228, 181 U.S.P.Q. 31 (CCPA 1974). Because of the rapid development of biotechnology, both the PTO examiner and patent applicant will be subject to the constant challenge to assess the issues of enablement and patentability from the perspective of the level of skill in the art at a particular point in time - e.g., as of the filing date (enablement); or at the time the invention was made (35 U.S.C. 103).

Enablement must be commensurate in scope with the claims. The decision Ex Parte Jackson et al., 217 U.S.P.Q. 804 (USPTO Bd. App. 1982) has a material bearing on the scope of claims that will be allowed in biotechnology - related inventions involving microorganisms. In this case, the Board of Appeals held that the disclosure was enabling only with respect to the claim directed to using the three microorganism strains that had been deposited, but was not enabling as to the process using the microorganism species generically. The Board reasoned that undue experimentation was required to discover other species not deposited capable of producing the novel antibiotic even in view of the taxonomic/functional characteristics described in the specification.

The basic issue in cases such as Jackson is undue experimentation. The PTO has the initial burden of giving reasons, supported by the record as a whole, why the specification disclosure is not considered enabling. Mere speculation will not suffice. Reasons and evidence sufficient to create a reasonable doubt as to the accuracy of a particular broad statement put forward as enabling support for a claim can take any of the following forms: (1) statements on their face contrary to generally accepted scientific principles, (2) teachings in pertinent references, (3) unpredictability of

chemical reactions. In re Marzocchi, F2d 220, 169 U.S.P.Q. 367 (CCPA 1971). Once the examiner has advanced a reasonable basis for questioning the adequacy of the disclosure, it is incumbent upon applicant to rebut that challenge. For example, an affidavit from a qualified expert could detail the procedures that were followed using the specification disclosure as a guide and the results that were obtained. The scope of the affidavit would depend on the scope of the disclosure and the scope of the claims.

Best Mode.

In the absence of evidence of concealment (accidental or intentional) the patent examiner will assume that the best mode requirement has been satisfied. In re Sherwood, 204 U.S.P.Q. 437 (CCPA 1980). Best mode is a separate and distinct requirement from the enabling requirement. In re Newton, 414 F2d 1400, 163 U.S.P.Q. 34 (CCPA 1969). It has been suggested that the best mode requirement might necessitate the inclusion on a greater degree of specificity than that necessary to comply with the enablement requirement of Section 112. In re Boom, 439 F2d, 169 U.S.P.Q. 231 (CCPA 1971). If the best mode contemplated by the inventor at the time of filing the application is not disclosed, such defect cannot be cured by submitting an amendment seeking to put into the specification something required to be there when the patent application was originally filed. In re Hay, 584, F2d 917, 189 U.S.P.Q. 790 (CCPA 1976).

Deposits.

When the invention depends on the use of a microorganism or like material, such as a naturally occurring microorganism, cell line, virus or plasmid, isolated and used to produce a new product, which is not known and readily available to the public and cannot be described by words alone, applicants must take additional steps to comply with the requirements of Section 112. One way to satisfy those steps is to comply with the guidelines in Section 609.01(p) of the Manual of Patent Examining Procedure for "(c) Deposit of Microorganisms." As the Court pointed out in Argoudelis, these deposition procedures are not the minimum statutory standards, but merely one way the requirements of the first paragraph of 35 U.S.C. 112 can be met. However, like the decision of whether a deposit is required or not, variances from the published guidelines should be undertaken only by mistake or after a careful assessment of the risks attendant a holding that the deposit does not meet the requirements of Section 112.

The deposit must be made no later than the effective filing date of the application to satisfy the requirements of Section 112. Ex Parte Lundack, (Unreported Board of Appeals decision dated August 21, 1984; Appeal heard at CAFC on May 10, 1985). (Subsequent to this presentation, In re Lundak was decided by the Court of Appeals for the Federal Circuit on

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September 16, 1985, 227 USPQ 90). The Court reversed the Patent and Trademark Office indicating that a deposit in a public depository is not required at the time of filing a patent application.) The deposit must be made in a depository affording permanence of the deposit and ready accessibility thereto by the public if a patent is granted. Clearly, any International Depository Authority approved by the World Intellectual Property Organization under the Budapest Treaty (1977) meets the requirements for an appropriate depository. Generally, a university laboratory facility would not be considered to satisfy the requirements for an appropriate depository.

The deposit must be made under conditions that all restrictions on the availability to the public of the deposited culture will be irrevocably removed upon the granting of the patent. The Board of Appeals decision in Lundack indicated that a period of deposit equivalent to that required under the Budapest Treaty (i.e., stored for a period of at least five years after the most recent request for the furnishing of a sample of the deposited microorganism and, in any case, for a period of at least 30 years after the date of deposit) was a reasonable approximation of the requirement for an assurance of permanent availability of the culture to the public. Appropriate documentation in the form of a copy of the contract with the depository and/or an appropriate statement to show that the guideline requirements have been satisfied is required.

A number of questions have arisen over the years concerning specific procedures and policies relating to deposits. Some of the issues and policies raised include: the need for viability testing for deposits; the maintenance and replacement requirements for deposits; the effects on a patent when a deposit is not maintained; what restrictions can be placed on access to deposits such as a requirement that the patent owner be notified when a sample of the deposit is provided another party; public vs. private depositories owned by the patentee; requirements for notification of the depository when the patent issues; when a deposit is required vs. public availability of the substance to be deposited; whether a deposit must be maintained after the substance becomes widely available or the technology is such that the deposit is no longer required to support the disclsoure; and whether and where seeds and plants (outside the PVPA) can be deposited. The PTO is reviewing questions such as these and plans to seek public comment on an appropriate expanded deposit policy.

As you can see, there are many activities and certain issues in the biotechnology area that directly affect the protection of plant and agricultural products and processes. The policy of the Administration and most of the legislation enacted by Congress in this and other areas is clearly towards the strenthening of intellectual property protection because of its importance to the development of the technology and to U.S. competitiveness. At the same time, care must be taken in the emerging biotechnology field to properly address the legal requirements and issues involved when seeking and enforcing patents.

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Chapter 22

Protection of Biotechnology in Agricultural-Related Inventions

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The availability of recombinant DNA techniques offers patenting opportunities hitherto unavailable in agricultural products and processes. The types of inventions protectable, scope of protection, and special requirements such as deposit will be discussed. The effect of genetic alteration of metabolic requirements, pest resistance, and disease resistance on the value of patent protection for traditional agricultural products, and the importance of protection of genetic engineering inventions in accelerating the growth of a para-agricultural industry will also be considered. Patent coverage outside the U.S. is also available.

Biotechnology means many things to many people and really includes such ancient arts as the making of wine and beer, fermentation of soybeans to make sho-yu, and classical plant and animal breeding techniques. All of these have yielded useful, productive, and even spectacular results. This paper, however, focuses specifically on what the underwriters, investment houses, and venture capitalists have found so glamourous, namely recombinant DNA technology and hybridoma technology. Neither of these "glamour" fields even existed fifteen years ago, and indeed, each represents a quantum leap forward in capability to manipulate the biomass.

Over the last decade, these two technologies have matured into a pattern into which most innovations needing protection can be nestled. Hybridoma technology is the simpler of the two to handle in terms of the parameters of intellectual property protection, and perhaps the less applicable to agricultural products. The technology is by now familiar to most, at least in broad outline; its applicability is really confined to vertebrate species and has to

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do with refinements on the ability of the immune system to generate specific immunoglobulins against specific antigens. A hybridoma is, as the name implies, a hybrid between a cell line which is inherently immortal such as a tumor cell and an antibody-secreting lymphocyte. The lymphocyte is obtained by immunizing a sheep or rat or other animal with the antigen to which antibodies are desired. By immortalizing the population of antibody-secreting peripheral blood lymphocytes or spleen cells, one is able to choose and perpetuate one out of the complex mixture of antibodies raised against this antigen for perpetual production. Because a single colony of immortal hybridoma produces the chosen antibody, the resulting antibodies are called "monoclonal" antibodies or "Mabs."

Monoclonal antibody technology has really rather limited application in agriculture. In general, the uses found for Mabs have been confined to employment in diagnostic tests for various diseases and to a more limited extent in passive immunotherapy. The only clear application, therefore, to agricultural problems relates to animal husbandry and the diagnosis and control of diseases in domestic animals. This has not been a particularly popular line of research as far as I know, and the protection of this technology raises no particular problems unique to agriculture.

Recombinant DNA technology, on the other hand, has wide and substantial application to agriculture, a somewhat more complex pattern basis for innovation, and when applied to agricultural concerns can raise issues which are not found in more conventional applications of this methodology.

Recombinant DNA inventions can generally be placed in a conceptual context illustrated by the diagram in Figure 1.

As shown in the diagram, the desired "product," which is put in quotes for reasons that will become evident, of the technology is a single Protein which has some desired property. The Protein is, of course, ordinarily produced by some organism or is modeled on one which is so produced and is encoded in the DNA of its normal source. The usual progression is to determine at least partially the amino acid sequence of the protein, which permits construction of oligonucleotide probes useful in identifying which portions of a DNA library are in fact those responsible for encoding the protein. The DNA library can generally be constructed either by chopping up the genome of the native organism or by reverse transcription from the collection of messenger RNA's in cells which produce the protein. (Alternatively, the library can be directly constructed in expression vectors and production of the desired protein detected using antibodies raised against it, eliminating the need for oligonucleotide probes.)

In either case the library will contain, in addition to the desired coding sequence, a multitude of irrelevant DNA's which need to be discarded. There is a slight exception to this in the case of some cells such as specialized cells responsible for production, for example, of -globin or, to a lesser extent, in producing insulin where the desired DNA predominates. (These were the first problems attacked by genetic engineers, but for the proteins of interest today it is more common that their encoding sequences

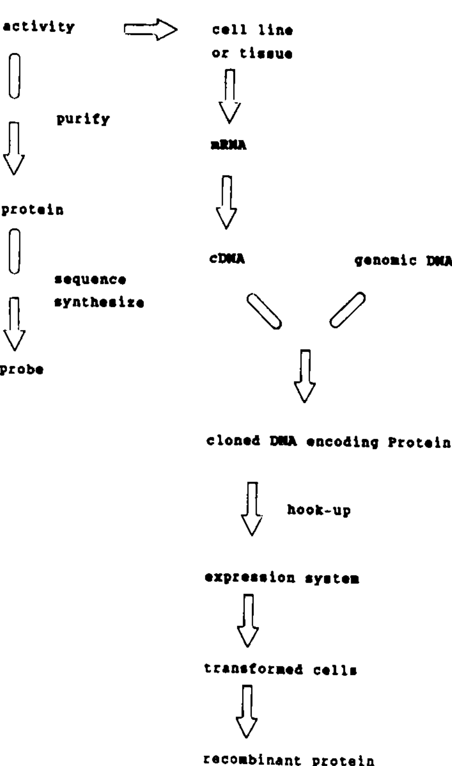

Figure 1. Pathway to recombinant DNA inventions.

represent only a minor portion of the library.) Anyway, the probe is used to fish out the desired sequence; the sequence is then usually amplified by transformation into a bacterium; the amplified sequence is hooked up to DNA which controls its expression in a desired host cell or organism; and the transformed host is then used as a production factory for the desired Protein.

Initial Applications of Biotechnology to Agriculture

The initial applications of this technology, and those upon which the high tech glamour companies such as Genentech and Cetus have achieved their high profiles, have been production of proteins as commercial products for sale to the consumer. Some of these products are not irrelevant to agriculture. While many are pharmaceutical compounds such as insulin, growth hormone, interleukin-2, and tumor necrosis factor, many are specifically directed to agricultural application. The most notable example is perhaps bovine growth hormone, in which a number of companies are interested for its obvious application - bovine interferon, which presumably would be useful in protecting animals from viral diseases; and in the coat proteins for various viruses which infect domesticated animals for use as vaccines. As applied to plants, exotoxins for use as pesticides can be made available in practical quantity. As substrates for intellectual property protection, the innovations relating to production of these products are really no different from those related to what might be considered standard biotechnology inventions.

Protection of End Products

There are, however, other portions of the diagram in Figure 1 which represent end "products" suitable for protection. One such class of invention, which is perhaps more uniquely related to agriculture but which can be dealt with using the same set of tools as other and familiar technologies, is the production of microorganisms which are useful in plant cultivation (designated "transformed cells" on the diagram, although microorganisms altered in other ways also should be included). The most notable example relates to nitrogen-fixing bacteria which are known to be symbiotic with legumes. This symbiosis formed the basis for the well-known technique of crop rotation to replenish the nitrogen in the soil. Clearly a potential exists for creating a microorganism which is superior in this property through a knowledge of the enzymic systems responsible for it and therefore cloning and expressing the genes encoding these enzymes, or by altering the symbiotic properties of these microorganisms so that they can coexist with plants other than legumes. Both of these approaches have been seriously considered and worked on.

Protection of Intellectual Property

For the above examples, at least one of the commercial products is either a protein or a microorganism. The issue now arises as to how best to permit the innovator, e.g., the developer of the bug for recombinant protein production or of the modified microorganism for N_2 fixation, to derive the benefits of this work. In the jargon of this field, this work is intellectual property, and there

are four traditional ways to protect intellectual property: patent, copyright, trademark, and trade secret.

Of these four, trademark is something of a wild card in that it is available to anyone who commercializes anything (whether it has an intellectual content or not). It's a name, like Ortho or Black Flag, which identifies a reliable commercial source for the goods or services so labeled. If the microorganisms or proteins are marketed, they can, of course, be trademarked; if the services of applying them to a particular set of circumstances are marketed, they too can be service marked. For example, Humulin is the current mark used by Lilly for human insulin; Genentech is using Protropin (not "Shortstop") for human growth hormone. Selection of a trademark can be most important; for example, patented drugs which acquired a strong trademark during their exclusivity maintain that exclusivity fairly effectively for an extended period long after the patent has expired and generic producers have entered the field. It is presumably the trademark which helps to accomplish this.

Copyrights are a fairly easy discard in this context. This mode of protection is designed to protect works of authorship for the expression contained therein. It does not preclude independent development of even the exact same thing. While it was suggested, perhaps even seriously, fairly early on that DNA sequences could be copyrighted, and learned presentations were given as to the advantages of using the protection of a copyright law as opposed to the patent statute in protecting these sequences, this represents little more than a gimmick, mainly because of the lack of protection against independent development.

This leaves trade secret and patent protection, both of which are appropriate to protection of inventions which involve the material universe. I would suggest that while trade secret protection may be appropriate for certain aspects of the manipulations which are conducted by the producer of the protein or the microorganism, it cannot be relied on as the only means for assuring exclusivity in an effective portion of this field. Certainly trade secrets cannot protect any product which can be reverse-engineered.

There are some good reasons that trade secret protection is inappropriate here. The first is practical. Most of the developers of innovations in this biotechnology are basically highly-trained academic people who have not been sufficiently brainwashed to keep their mouths shut. This may partially disappear as this industry progresses to a more mature(?) self-aggrandizing(?) member of the commercial community.

A more serious deficiency in trade secret protection, which is not curable by muzzling employees, is that, like the copyright law, it is no protection against independent development of the equivalent or exact same thing. Therefore, even if company A's process for manufacturing protein X is effectively hidden, another worker or another company is perfectly free to develop its own process for producing protein X and, indeed, can use the precise process of company A if it is in fact developed without access to the trade secrets of company A. Even the most casual observer of the biotechnology field is aware that everyone is pretty much working on

the same thing and the probability that company B will fail to come up with something viable in producing protein X legitimately in competition with company A is pretty remote.

Closely related to this is the fact that trade secret protection is inherently lacking in generic coverage. There is no way to bootstrap a particular invention into one of broader scope. That is to say if one makes, for the first time, bovine growth hormone by a secret process, the secret covers only the process actually used and does not cover alternative processes which also may succeed, perhaps better, in producing the same protein (or the same process, independently developed). If one wishes protection and exclusivity with regard to any recombinant process for producing bovine growth hormone, or even any class of recombinant processes for making it, one cannot rely on trade secret protection unless one is certain that one has the only workable process within this genus (and that this workable process is not readily independently discoverable).

In short, trade secret protection is appropriate only for tricks of the trade that it seems unlikely a competitor would discover independently. There are precious few of these in the biotechnology field, although it seems prudent to remain on the lookout for them. To take a rather absurd example, if it turns out that rinsing the fermentation vats with Calgon dishwasher detergent, but not with Electrosol or Tide, diminishes the probability of phage infection of the production strain, it might be wise to keep that a trade secret. On the other hand, if it appears that a defined class of detergents works, it might be wise to obtain patent protection on the generic theme of using a particular class of detergents.

Patent Protection

This leaves patent protection. To the extent that such protection can be obtained, this is clearly the most desirable. It provides seventeen-year exclusivity in the United States once the patent issues and the subject matter becomes public knowledge. Abroad, patent terms vary, but twenty years from the date of filing (not from the date of issue) is a commonly encountered term in most countries. Publication of the patent application occurs before issue, but generally damages for infringement are retroactive to publication if certain precautions are taken, and provided the patent eventualy passes to issue.

The clear advantages of patent protection are statutory exclusivity, the ability to extend a particular embodiment to a more generic scope of protection, and the ability to exhibit this protection in public for the benefit of investors. The disadvantages are related to the expense and risks in obtaining this protection.

Cast in the overall cost of a research and development program, the expense is minimal. Dollar figures are extremely difficult to assess in generality. The cost to prepare a typical biotechnology application by an outside attorney is of the order of several thousand dollars, but the real unknown lies in the cost of prosecution, which often varies directly with the scope of the claims sought. One can consider such imponderables as whether it is less expensive to use a shotgun approach to obtain individual

patents on a variety of species within the same genus or to strive for an umbrella patent. For example, if one obtains a particular hormone X with a specific amino acid sequence, should one attempt to cover in the same application all biologically active permutations on the amino acid sequence of X, or should one file applications on the specific amino acid sequence of the first cloned X, and on those later permutations which are found to be active as they materialize? Should one attempt to cover only the particular nitrogen-fixing bacterium which one has obtained to grow on, say, asparagus; or should one attempt to claim any nitrogen-fixing bacterium which thus grows?

Besides expense, potential validity or invalidity needs to be considered. For example, in the recent infringement litigation involving Hybritech and Monoclonal Antibodies, the federal court for the Northern District of California held the patent claims in suit invalid on a number of grounds, one of which was anticipation by others. Claims covered an immunological sandwich assay wherein the improvement was the use of monoclonal as opposed to polyclonal antibodies. Whether the claims would have been considered invalid, for example, for obviousness, had particular monoclonal antibodies used in a particular immunoassay been claimed rather than trying to cover the broad sweep of such applications is, of course, a matter of speculation. But clearly such claims would have had a far greater chance of surviving the test of direct anticipation.

Other than expense, the risks of obtaining patent protection are few. Of course, one must enable others to practice the invention as part of the bargain to obtain temporary right to exclude others from doing so; and for patent applications made abroad, publication occurs before protection is granted. But sometimes the publicity is the name of the game, and one would like to impress investors or licensees with the scope of potential protection.

There is a unique factor in biotechnology cases which involves both expense and risk. The Patent Office requires a "deposit" under certain specified conditions, including public access to the deposited organism or sample after issuance of a U. S. patent, for inventions claiming products of transformed cells or the microorganisms or cells themselves. For reasons that are unclear, the actual practice of the Office appears to vary, especially where the claims are drawn to methods using particular cell lines or organisms.

With the holding in In re Lundak (CFC 1985) 227 USPQ 90, the necessity of completing the formalities of the deposit at a recognized Depository, such as the American Type Culture Collection, prior to the filing of the U. S. patent application has been obviated, thus removing a major nuisance factor which led to many a torn hair. However, deposit will still be required in many cases during prosecution, and the outright expense is formidable. Each deposited microorganism or cell line costs approximately $500 for the deposit alone. An additional $300 is needed if the applicant wishes to be notified of others requesting the deposit. For various reasons, including perhaps incomplete DNA fragments on a number of deposits and attempts to widen the scope of the application,

several deposits may be thought advisable for a single case, which adds considerably to the cost.

Worse, however, is the risk factor; it relates to unauthorized use of the deposit by a requester. Because of the best mode requirement of 35 USC 112, it is almost frivolous to deposit any other organism for producing a claimed protein, say, except the best one. But all that the best organism comprises may not be relevant _per se_ to the invention. In the most obvious case, if the desired product is a growth hormone for chickens, the invention really resides in obtaining the DNA sequences and hooking them up for expression. But one is required to deposit one's best producing transformed cells, while the production characteristics of this cell line are not necessarily determined, and probably are not, by the DNA encoding the desired protein. Rather they are determined by the hardiness of the cell line or organism itself and the fine-tuning of its control sequences. These may be valuable assets which the patentee wishes to protect. While the patentee may also be able to get a claim on the host cells independent of the transfected DNA, and thus on a theoretical basis prohibit others from using it, there _is_ the possibility of illicit use when the only practical way for the potential infringer to obtain this host is in fact from the deposit itself.

The other risk involves a potential for narrowing the interpretive scope of the claims. While there are no decisions yet on point, it is perhaps a reasonable argument that if the only enablement of production of protein X is the deposit of the producer, and if the claim to recombinant protein X is really a process claim (to distinguish it from protein X purified from native sources), then one might be reasonably limited to the process actually enabled, i.e., production by the deposited producer itself. It is not clear at present whether this result will ensue, and indeed it is to be hoped not, but it can certainly be argued.

While the same burden is on everybody, it is an unnecessary one. I cannot see how the Office can find a legitimate basis for it in the law. The citation most frequently given is, of course, _In re Argoudelis_ (CAFC 1970) 168 USPQ 199. The other two relevant cases are _In re Feldman v Aunstrup_ (CCPA 1975) 186 USPQ 108 and _Ex parte Jackson_ (Bd of Appeals 1982) 217 USPQ 804. None of these cases relate either to hybridoma or to recombinant DNA technology. All of them relate to organisms which are isolated from nature through a selection process. The case most commonly cited by the Office, _Argoudelis_, does not relate to _whether_ the deposit is required; only to what must be done when it is decided that a deposit is required. The holding of that case is that _if_ a description in writing is inadequate to describe the invention, _then_ a deposit may be used to satisfy the enablement requirement.

The decision in _Jackson_ takes the peculiar position that, as a matter of law,

> "a unique aspect of using microorganisms as starting materials is that a sufficient description of how to obtain the microorganism cannot be given."

The Board appears to cite _Argoudelis_ as _res judicata_ in this respect. There are, of course, two things wrong with this. First,

Argoudelis said nothing of the kind. Second, this was not a legal question but a question of fact. What the facts were in 1970 at the time Argoudelis was decided are not necessarily what the facts were in 1982 when Ex parte Jackson was decided, or what the facts are now. That notwithstanding, recombinant microorganisms and cell lines and hybridomas are not organisms isolated from nature and are not addressed by the holdings in any of these cases.

There are additional problems and limitations involved in obtaining patent protection on biotechnology in general, including matters such as determination of inventorship, the level of description required, and so forth, but these tend to be of interest mostly to the specialists. What one can say in general is that at least the products of recombinant technology so far discussed are clearly statutory subject matter, at least in the United States. They are also generally patentable elsewhere in the world, with a few notable exceptions such as countries which do not permit patenting of products of chemical processes. However, the major jurisdictions, Europe, Japan, and the United States, do not offer any notable subject matter hurdles in this regard (except that mammalian cell lines are not patentable in Japan). The Chakrabarty decision in the United States disposed of any question as to whether microorganisms are patentable, and the Patent Office has not taken any umbrage at attempts to patent cell lines.

Agricultural applications uniquely offer the prospect of seeking patent protection for higher plants and for higher forms of animal life. It has clearly been shown to be possible to manipulate genetically whole mammals, as typified by the transformation of mouse embryos with rat growth hormone as reported in Science in 1982. Furthermore, while the technology is not by any means as highly developed as that for bacteria, it appears possible to obtain genetic alterations in whole plants using recombinant techniques and transformation, at least in some species. One then needs to consider whether the resulting animals and resulting plants are also patentable subject matter.

With respect to higher animals, the position of the Office is that the Chakrabarty decision was not determinative for them, but only for microorganisms. It seems to reside in the general discomfiture with coming ever closer to manipulating the genetics of people. Certainly there is no logical reason from a statutory interpretation standpoint to draw the line at microorganisms. Curiously, an attempt in the early 1970's to obtain a patent on dwarf chickens which nevertheless laid normal size eggs, and thus required less food for the same egg production, ended in failure. The CCPA in In re Merat (CCPA 1975) 519 F2d 1390 refused the patent on the grounds that the application was nonenabling, and never reached the question as to whether the chicken could have been patented had the description been improved.

For plants, until recently, higher plants - with a minor exception of Irish potatoes, Jerusalem artichokes, and first generation hybrids - were held to be unpatentable subject matter by the Patent Office. However, a recent Board of Appeals decision, Ex parte Hibberd, et al., has clearly held that they are. Probably no one is as nervous about manipulating the genetic structure of

plants as a disturbance of the handiwork of God (for reasons that are not apparent), but perhaps because of this there are alternative means of protection available. The Office had taken the position that since Congress has offered alternative means of protection for certain kinds of plants, it intended these to be the exclusive means of protection for such plants. These protection systems are still viable and of interest, as they differ in scope from the protection offered by utility patents.

The Plant Patent Act (35 USC 161-163) was passed in 1930 to protect plant breeders. It covers only plants which can be asexually propagated, and is administered, as the code title would imply, by the same Patent and Trademark Office which handles the granting of utility patents. The other relevant act covers sexually reproducing plants and protection is formalized not by the Patent Office but by the Department of Agriculture. This is the Plant Variety Protection Act (PVPA) (7 USC 2321 et seq.). Since the Plant Patent Act excludes tuber propagators from protection and the PVPA excludes first generation hybrids from protection, these were the only higher plant species for which the Patent Office saw fit, prior to Hibberd, to grant utility patents.

The scope and limitations of protection afforded by these alternatives are clearly set forth in the statutes. The following is a very brief outline of similarities and differences in comparison to standard utility patents.

The Plant Patent Act consists of only four sections. 35 USC 161 says that whoever invents or discovers and asexually produces any distinct and new variety of plant, other than either a tuber-propagated plant or a plant found in an uncultivated state, may obtain a patent on it. It is to be noted that the mere discovery of a product of nature is patentable specifically under this section. Asexual reproduction is required. Section 162 loosens up the enablement requirements considerably and specifically states that no deposit is needed. Section 163 permits the patent owner to exclude others from asexually reproducing the plant or from selling or using plants thus reproduced. What this implies is that independent development of a plant to accomplish the same purpose where the other plant was not physically derived by asexual reproduction from the patented plant line is not an infringement. It has been so held. This could be a serious problem for genetic engineers. Independent development of an asexually reproducing plant by recombinant techniques should be well within the realm of possibility.

The Plant Variety Protection Act is somewhat more formidable, but nevertheless some summary distinctions can be made. First, it protects only plants which are reproduced sexually, and provides for obtaining a certificate from the Department of Agriculture which provides an eighteen-year period during which the holder may prohibit others from using seed labeled "unauthorized propagation prohibited" to produce other seed for marketing, selling or offering the plant for sale, importing it into the U. S. or exporting it, or inducing a third party to do any of the above. However there are a multitude of exemptions under which these acts are not prohibitable, including the use of the variety in the making of

still another improved plant and some rather complex considerations involving growth for food and harvesting seeds for a second crop. Again the protection appears directed to the specific plant line itself and not designed for generic coverage of plants with a specific desirable feature. As for the Plant Patent Act discussed above, the scope of protection under the PVPA is by no means identical to the scope of protection for utility patents.

There are a limited number of issued patents and published patent applications relating to the use of recombinant DNA techniques in higher plants or in organisms which infect or otherwise are associated with them. It is expected that with the *Hibberd* decision, increased use will be made of the utility patent system to obtain coverage for plants *per se*, despite the alternatives provided by the Plant Patent Act and by the PVPA.

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Chapter 23

Public Policies for Avoiding Mistakes in Biotechnology Developments

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Biotechnology developments promise extremely significant benefits. But if we are not careful, we can also make extremely significant mistakes. These mistakes fall into one of four categories: 1) identifying problems when they do not exist, 2) not identifying problems when they do exist, 3) unreasonably suppressing benefits because of excessive concern about problems that might exist, and 4) failing to promote benefits that could exist.

The challenge for public policy is to develop a development and review process which minimizes all four types of mistakes. This process must exist in an environment of substantial scientific and social uncertainty. If society develops a process for avoiding the second type of mistake efficiently, it will reduce both types of uncertainty, and be in a much better position to avoid the other three types of mistakes as well.

Biotechnology may be the most important scientific development of this century. It offers the prospect of creating almost unimaginable benefits. To the sick, it offers new cures for dreaded illnesses. To the farmer, it offers higher production, lower costs, and more valuable crops. To the industrialist, it offers cheaper production of basic raw materials and possible creation of entirely new commodities. It offers opportunities to destroy toxic as well as conventional pollutants, reduce the use of hazardous substances such as pesticides, and otherwise enhance our ability to protect and restore the environment.

The biotechnology revolution shares many of the same characteristics as the rapid development of organic chemicals around the turn of the century. That revolution also brought immense benefits to our society. It created new products, new drugs, and new processes that have made our life immeasurably better. Few would argue that we would have been better off without the organic chemical revolution. Even fewer could even imagine what life would be like had we not.

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Yet, we are now painfully aware that this revolution did not bring unmitigated benefits. As we wonder what to do with our hazardous waste dumps, our contaminated groundwater supplies, and our toxic air pollutants, many wish that we had managed aspects of this revolution better. Some see a similar two-edged sword with biotechnology: in the enthusiasm to reap the benefits of this new technology, we may be creating a new legacy of problems for future generations.

But one should not push the analogy between biotechnology and past scientific changes too far. There are important differences. The fact that we are dealing with basic biological processes--the essence of life itself--sets this technology apart from past advances in chemistry and physics.

In addition, this technology has emerged during a time when society takes a much different view toward risk than it did 50 or 100 years ago. New views toward science, and toward the rights of innovators and producers have also evolved. Many of these different views have been incorporated in a series of laws, environmental requirements, regulatory processes, and court decisions, that distinguishes the social environment in which this innovation is being introduced from any that has greeted other innovations.

What Will It Take To Do It Right?

The fundamental question is, what will it take to "do it right"? Have we learned anything from past scientific advances and from the tumultuous years of the last decade that can be applied to development of this technology? Doing it right does not mean encumbering the technology with deadening government controls. Doing it right means maximizing the benefits that the innovation can bring while minimizing its accompanying risks. If we do not do it right, society can experience some substantial and ultimately very costly mistakes.

To do it right, we must try to avoid making four types of mistakes. There is a Type I mistake--reacting to problems that do not really exist. The converse of this is a Type II mistake--not identifying problems that do exist. The Type III mistake is (unreasonably suppressing the development of the technology because of) excessive worry about what problems may exist. Any the Type IV mistake is failing to promote the full range of social benefits that the technology could provide if it is developed.

What Is The Challenge To Society?

The challenge for society is to establish a decision making process--a process that encompasses research, production and regulatory elements--that will minimize these four types of mistakes. And while such a process would be difficult to establish even under the best of circumstances, we must develop this process in an environment of substantial uncertainty.

Everyone in this audience is certainly aware of the scientific uncertainty that is inherent in any major breakthrough like biotechnology. Researchers, of course, focus primarily on the uncertainty surrounding the ultimate success of their experiments.

In Biotechnology in Agricultural Chemistry; LeBaron, H., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

Scientific uncertainty also includes the questions of whether the new products will create undesirable "side" effects.

Scientists often are less familiar with the broader problem of social uncertainty that has substantially increased in recent years. Social uncertainty encompasses all the legal, social, political and perhaps even religious factors that are likely to have a substantial impact upon the success of an innovation.

The scientific and social uncertainties are closely interrelated. Reducing scientific uncertainty is usually a necessary, but not sufficient, condition for reducing social uncertainty. An effort to reduce both types of uncertainty can help minimize the risk of making the four types of mistakes listed above.

Reducing Scientific Uncertainty

Reducing scientific uncertainty is an obvious precondition to reducing the Type I error mentioned above--reacting to problems that do not exist. The short history of the biotechnology revolution contains a truly remarkable effort to reduce such errors. This was the Asilomar Conference, and the moratorium on biotechnology research resulting from it, that occurred during the mid-1970s. The conference was held because some prominent scientists in the field feared that biotechnology might produce very serious problems. The research moratorium provided time to assess how serious these problems might be, modify the direction of research efforts to avoid the most serious problems, and create a mechanism for ensuring that appropriate care was taken in conducting the research that did occur. This conference and moratorium were voluntarily undertaken by the scientists involved in the research. It was a truly remarkable--and successful--step. Its major conclusion was that the problems associated with biotechnology research were probably less serious than some feared, and that mechanisms could be adopted for reducing any risks to very low levels. The Asilomar Conference substantially reduced the risk of Type I errors--at least for the research phase.

Avoidance of Type I errors during the research phase, however, does not necessarily ensure that they will be prevented during the development and commercialization phases. Clearly some products of the biotechnology revolution will create very little risk when released into the environment. Most nonliving products are an obvious example. But some of the procedures adopted to reduce risks in the research phase may not work as effectively in development and commercialization. The containment strategy is one. It is relatively easy to devise means for containing organisms in a laboratory. It is much more difficult to devise techniques for containing organisms that are released into the environment, or to destroy them once they are released.

One of the most obvious steps that society can take to avoid Type I errors is to develop a system for classifying biotechnology products that clearly indicates which products are highly unlikely to create problems, which deserve some review, and which deserve careful review. Such a system would substantially reduce uncertainty for the developers and the regulators of the products, and would prevent unnecessary resources being directed at reviewing products that are unlikely to cause any problems.

Avoiding the Type II error--not identifying problems that do exist--is a more difficult task. It requires the development of a set of methodologies for understanding and assessing the types of risks that might be created by biotechnology developments. These methodologies do not now exist and developing them will be very difficult. It will require a sophisticated understanding of micro macro biological interactions that does not now exist.

Risk Assessment

Despite many years of effort, the current ability of scientists to predict the risks associated with a new chemical remains relatively limited. Predicting the risks associated with new combinations of genes or new life forms is substantially more difficult. With chemicals, scientists can at least agree on a reasonable set of tests that can be used to assess a chemical's risks. No such "base set" exists for the products of biotechnology.

Identifying such a base set of tests and developing appropriate risk assessment methodologies ought to be a major concern of everyone involved in the biotechnology revolution--including the scientists doing the research, the companies attempting to commercialize the products, and the regulators and public interest groups concerned about avoiding these problems. Professor Simon Levin and Mark Harwell of Cornell have outlined elsewhere some of the research that needs to be done to develop adequate approaches to assessing and reducing potential risks associated with biotechnology developments.

Predicting Environmental Fate

The first of these needs is the development of accurate methodologies for predicting the transport and fate of the products that are introduced into the environment. Such a prediction methodology needs to be concerned both with the survival and growth of living organisms introduced into the environment and how these are affected by environmental conditions, as well as with plasmid transfer--the ability of gene combinations to be introduced into new populations. Both of these are extremely difficult to predict, because whatever is introduced into the environment may undergo evolutionary changes once it is there.

A good model on transport and fate would allow estimates to be made of environmental exposure to potentially harmful organisms. But it is still necessary to develop methodologies for predicting what the effects of that exposure will be. With biotechnology, the concern is not only with how a particular organism will affect some other particular organisms, but how the introduction of a new strain will affect the dynamics of particular populations, community structures and ecosystem functions. The effects of concern are not only the direct effects, as has normally been the case in assessing the risk of chemicals, but the indirect effects on populations and ecosystems. The existing science of ecology is ill prepared to make such predictions at either the micro or macro level.

It seems unlikely that society will be willing to delay the introduction of the fruits of biotechnology research until adequate

methodologies for predicting transport, fate, and effects have been developed. But there are some ways to reduce Type II errors while these methodologies are being developed. These are the other needs Levine and Harvwell identify. One is to establish systems for monitoring the transport, fate and effects of introductions so that we can tell when something is not going as expected. A sensitive monitoring scheme might well allow action to be taken to avoid a problem before it is too late. Again, however, creating such a scheme is not a trivial undertaking. There are fundamental questions of what is monitored, where it is monitored and how it is monitored.

Containment

A second step is containment. One reason why research has been allowed to continue in the laboratories is that this research is done in such a way as there was little risk of release--the experiments are contained. Can such containment systems be developed to control released organisms? If so, the risk of the releases creating problems--that is the risk of a Type II error-- would be reduced. Fortunately, there is some economic incentive for private firms to develop containment systems, because the more they are able to contain their releases the more demand there will be for their products.

Mitigation Strategies

A third step is developing adequate mitigation strategies. What can be done to correct a problem if it occurs? What can be done to control the spread of a microorganism once the monitoring system indicates it may be getting out of control? The certainty that it is possible to mitigate any mistakes would clearly reduce the anxiety of experiencing Type II errors associated with any proposed release.

The primary fear of many of the scientists and firms involved in biotechnology revolution is obviously the Type III mistake--that there will be an unreasonable suppression of the development. But avoiding this mistake is closely tied to our ability to avoid a Type II mistake. The more confident society is that releasing the fruits of biotechnology experiments will not create unexpected problems, the less likely it is to impose oppressive restrictions on this development. The key question is whether we can be confident of avoiding Type II errors without implementing such controls.

Some scientists have suggested the way to avoid Type III errors is to hold another Asilomar Conference. Get a group of bright scientists together, let them address this problem, and then let them tell the world that there is no problem. Under this approach the world will then relax and avoid imposing any possible constraining regulations.

But the Asilomar method is not likely to be as successful at the release stage as it was at the research stage. At the research stage, society was willing to accept the scientists' assurance that the experiments could not create problems because even if the organisms happened to be harmful, they would not be allowed to escape from the laboratory to affect the environment. But the release

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stage is obviously quite different. Here the intention is to release organisms with the specific purpose of affecting the environment. The assurances given at the research stage can no longer suffice. As a result, non-scientists are likely to take a much greater interest.

The Type III mistake will only be avoided as a result of an active dialogue about the potential benefits, risks, and proposed uses of biotechnology. This dialogue has to incorporate diverse professional perspectives, interests, and social perspectives. It has to include the politicians as well as the scientists, the public interest community as well as businessmen, the fabled tennis shoed activist as well as the government bureaucrat. All have to start talking together and agreeing on what the issues really are, where the problems do really exist, and where they do not exist. Once these agreements are reached they have to be communicated to the public at large. Initiating this dialogue and communication is one of the most important steps that the biotechnology community could--and should--be taking right now.

Falling into the Type III mistake could create serious costs of foregone opportunities for society. Without an effective dialogue involving all interests, the narrow yet valid concerns of one or two could stymie safe biotechnical advances. Concern stems not just from the "environmental" community; biotechnology has aroused some ethical and religious issues that could coalesce into an almost unbeatable obstacle to continued development.

Finally, avoiding the third mistake will be much easier if we set up a process for avoiding the Type IV mistakes--that is, not fully realizing the benefits that biotechnology could bring. Because not all of the developments promise commercial profitability, the free market may not ensure that all the possible benefits will be realized. There is a need to set up a process for identifying the social benefits that biotechnology can bring and for seeing which of these are likely to be commercialized and which are not. Identifying these benefits and initiating efforts to ensure that they are realized will make society more receptive to taking the risks that may also be associated with these and other biotechnology proposals.

These, then, are the four types of mistakes we may make as we begin to attempt to exploit and manage the revolution in biotechnology. Any one of them could be extremely expensive. Unfortunately, there is some evidence that we are in the process of making--or at least not protecting ourselves from making--all four simultaneously. The evidence to date unfortunately suggests that we may not yet have learned how to do it right.

Improving The Prospects We Face

Several different avenues may be advocated for attempting to improve the prospects we face. Scientists and entrepreneurs at greatest financial risk may argue that everything will be all right if the scientists are just allowed to do what they know is right. "Leave it to us" they assure the world, "for no one understands these issues better than we." Whether or not this argument is correct, it is probably irrelevant. A development as important as

biotechnology is not going to be left to the scientists; at some point, questions must be put to a broader social test. If they are not, it is likely to end up with some very contentious and ultimately destructive debate down the road. The reaction could well be an extremely frustrating and debilitating regulatory process.

A great deal of attention is already being given to what the regulatory process should be. And this is the second main approach to dealing with the problem: let government regulate it. But just handing the problem over to the regulators is not going to avoid all the mistakes. It may not, in fact, avoid any of them. Anyone who has been involved in the effort to regulate toxic substances or environmental pollutants knows how insensitive and inefficient such a process can be. Undoubtedly there will have to be some regulatory process, but it can be carefully designed with great sensitivity to avoid all four types of mistake.

The third avenue--establishing the dialogue described above--would help integrate the concerns of business, government, and citizens. The time to establish a dialogue is before developments proceed too far and debates become too contentious. This dialogue will be a very frustrating process. The scientists involved will be frustrated by what they perceive as stupid questions, and the non-scientists will be frustrated by what they perceive as naive answers. But such a dialogue, frustrating as it may be, is probably the best way of avoiding the four mistakes described in this paper.

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Chapter 24

Safety Issues in Perspective

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The appropriate level of regulatory scrutiny for field testing recombinant organisms should be based on our extensive experience with traditional agricultural practices. When safety issues are examined from a scientific point of view, it can be concluded that the risk from recombinant organisms is no different than the risk that has been associated with traditional plant or microbial genetic research and practices of many decades.

Plants or microorganisms that seem, from laboratory or greenhouse experiments, to be potentially useful in agriculture must be field tested to check the relevance of these results. There are innumerable cases of exciting greenhouse findings not reproducible in the field. All of the many changing environmental factors a plant experiences in the field cannot be replicated by greenhouse experiments. Therefore, small-scale field testing early in a project is essential if organisms are to be considered for use in agriculture. This has been the case for organisms not genetically altered as well as for genetically modified organisms.

Federal agencies are now deciding how to regulate recombinant plants and microorganisms to ensure that their release into the environment will not create health or environmental problems. This paper will address the potential for problems that may arise from releasing recombinant organisms. The chance of this technology producing a serious problem should be compared to problems we now accept from current practices.

PLANTS

An experiment to improve crop plants by adding foreign genes should have less of a chance for problems than traditional breeding experiments. Breeders routinely cross cultivated plants with wild, exotic relatives. Until the results of the cross have been

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analyzed, the breeder cannot predict progeny characteristics, which are the result of random mixing of thousands of genes. Breeders are not concerned that a plant derived from such a cross will become a problem weed because extensive, world-wide experience with these types of crosses has not produced plants that are difficult to control. Scientists know that weeds such as dandelion, pigweed, or kudzu are problems because they have a number of properties that enable them to predominate and compete against other plants in their environment. Such problem weeds probably require dozens, if not hundreds, of specific genes to maintain their "weedy" character. Therefore, many specialized genes are required to convert a non-weedy crop plant into a weed that would cause a serious problem.

Compared to breeding a crop with exotic species, a genetic engineering experiment with that crop is very specific. The introduced foreign genes probably are well characterized, plant modifications are not due to random mixing of genes, and the properties of the plant are quite predictable. There seems to be no chance that expression of several characterized genes added to a crop plant for the purpose of making it more valuable will create a weed that could cause problems of the magnitude of a kudzu. Even if several uncharacterized genes are added to the crop plant, the chance that they will interact in a way to make that plant become a problem weed should be sufficiently low to become a negligible concern.

Current practice with chemicals creates large populations of genetically altered organisms. For instance, herbicides added to millions of acres year after year produce mutant weeds that become resistant to the chemical. Similarly, insecticide use results in insecticide-resistant insects. Thus, chemicals cause uncharacterized changes in problem organisms. By comparison, genetic engineering will create characterized changes in safe organisms. There is no concern that genetic events from use of chemical pesticides will cause problems in environments lacking the applied chemical (pesticide), even though the genetically altered weed or insect readily move to chemical-free environments.

Many groups applying recombinant DNA technology to plants are trying to create plants which are resistant to pests or produce their own herbicides. Thus, future agriculture will be far less dependent on synthetic organic chemicals as pesticides and herbicides. Laboratories have introduced the *Bacillus thuringiensis* toxin into plants. This toxin is a protein that specifically kills certain caterpillars. It is nontoxic to man, bees, and other insects and has been a commercial product, applied to vegetables and forests, for many years. Unlike the situation with chemical insecticides, toxin-resistant insects do not readily appear. If the toxin gene is suitably expressed in plants, then such plants are potentially resistant to many troublesome insects. Other toxins specific to a different range of insects also are known. Such protein toxins will cause fewer problems to man and the environment than currently used chemical pesticides.

As is the case with traditional breeding practices, there will be problems caused by recombinant plants. Addition of a foreign gene may simultaneously give the plant an undesirable property (e.g. greater susceptibility to drought). Undesirable features usually are detected during experimental field trials. If these negative characteristics cannot be eliminated from the plant, the new variety will not be useful commercially. If a problem appears only after commercial introduction, the variety will rapidly be replaced by a new one. Breeders are trained to be alert for these types of problems. Because the genetic alteration in a recombinant plant is well-controlled, the likelihood of a problem is far less than in standard breeding practices.

MICROORGANISMS

Because microorganisms (bacteria and fungi) are invisible, can readily be transported, and are known to cause plant and animal diseases, there seems to be a greater concern for recombinant microorganisms than for recombinant plants released to the environment. In the case of microorganisms known to be pathogens, special care about release certainly is warranted; however, a recombinant pathogen probably is less dangerous than its unmodified parent due to the increased genetic load by the added recombinant gene. Regulations that satisfactorily govern release of pathogens (e.g. for biocontrol) should be relevant to recombinant pathogens.

There are no federal regulations that govern experimental release of native microorganisms that are considered to be safe. Since the turn of the century, hundreds of different microbial products have been sold. Therefore, thousands of experimental inoculants have been field tested. These inoculants include bacteria to fix nitrogen, algae to fertilize the soil, bacteria to stimulate plant growth, and fungi to increase phosphorus uptake by plants. In many cases, mutant strains have been utilized. There has not been a single problem that as gotten out of control through these practices.

A recombinant microorganism used for agriculture is expected to contain one or several characterized foreign genes. These genes should not create problem animal or plant pathogens (ones that could efficiently spread disease) from non-pathogenic microorganisms. Studies with animal and plant pathogens have shown that many specific interacting genes are required for the organism to damage its host, maintain pathogenesis genes and travel between hosts. It seems virtually impossible that an experiment designed to improve plant growth with microorganisms modified by recombinant DNA technology will give the microorganism all of these specific "pathogenesis" genes. With standard laboratory genetic techniques that have been in common use for decades, there has been no example of a safe microbe being genetically altered to become a problem pathogen.

Experience in the chemical industry has sensitized us to be alert for serious unexpected problems. Bhopal, Three Mile Island and Love Canal are examples. In the case of chemicals known to be toxic, a dangerous situation is potentially present during manufacture, use, transportation, storage or disposal of the chemical. In the case of recombinant microorganisms potentially useful to agriculture, there is no apparent danger; therefore, a spill or spread of the organism would not create a problem. Chemicals that may be very similar (analogs) to safe chemicals are known to be dangerous. In fact, analogs of metabolites can be among the most dangerous chemicals. Therefore, each new chemical should be tested for safety no matter what its structure is. However, microorganisms known to be safe have been mutated and have exchanged genes in laboratories and there has been no case of a man-made derivative of a safe organism becoming a significant problem. Thus, analogies of recombinant microorganisms with chemicals is not always appropriate when considering safety concerns.

Current technology with chemicals has caused genetic changes in microorganisms that have been detrimental. For example, herbicides applied to the soil provide a source of food for microorganisms that mutate to efficiently degrade the chemical and thus render it ineffective against weeds. Use of antibiotics has enriched the environment for antibiotic-degrading bacteria. Problems caused by these genetically altered microorganisms can readily be solved by discontinuing use of the chemicals. Many researchers hope that recombinant DNA technology will develop medical and environmental products to replace some of these chemicals and, therefore, to alleviate problems that such chemicals currently cause.

NATURAL SELECTION

A plant or microbe contains thousands of active genes. Every minute, phenomenal numbers of organisms undergo the natural process of mutation and exchange of genes. Organisms are continually being transported across ecosystems to new environments, and evolution slowly progresses as a rare organism increases its ability to survive and multiply. There is more and more evidence that genes have naturally crossed genus and even kingdom barriers. Adding man-made strains to our environment should have neglible impact compared to normal processes. The number of genetic alterations through scientific experiments are of much smaller magnitude than alterations occurring daily in nature.

As new selective pressures appear, an organism is either able to compete or is selected against. In cases when man adds a new chemical to the environment, the mere presence of that chemical can give an organism an "artificial" selective advantage. Examples include antibiotic and herbicide metabolism noted above. In the absence of such "artificial" selection (e.g. by a specific chemical), an organism survives because it is adapted to handle many varied selective pressures. Thus, a great many specific genes are

required by an organism to remain competitive in changing environments over a significant period of time.

Scientists have demonstrated that even a single gene not needed by a bacterium can give that bacterium a selective disadvantage. Over time, genes not helping a bacterium survive in the environment most likely will be lost through mutation. Those organisms which maintain a non-functional gene will be at a selective disadvantage relative to those that have lost the non-functional gene through mutation. Plants developed for increased yield through intensive agricultural practices are extremely debilitated in their ability to compete in the wild. Adding disease resistance properties or increased seed production through mutation and breeding have not given the crop an opportunity to become more adaptable. Each modification for man's use further debilitates an organism's ability to compete in nature. The eons of selection, mutation and recombination have given us organisms that are efficiently adapted. A scientist would have an extremely difficult time purposely engineering an organism to be better adapted to a natural environment. There is no basis for the belief that a plant or microorganism derived through genetic engineering will unintentionally become more competitive and create an environmental problem.

CONTAINED TESTS TO DEMONSTRATE SAFETY

In order to demonstrate unexpected problems from released genetically engineered (or any other genetic alteration) organisms, small-scale field tests are essential. Suitable laboratory tests to demonstrate that no unpredicted field problems will emerge with recombinant organisms are unlikely to be devised in the forseeable future. No greenhouse or laboratory tests are required (or have been necessary) to demonstrate safety of mutated or cross-bred organisms released for agricultural benefit. The researcher certainly is naturally interested in the outcome of any experiment, and experimental fields are routinely monitored for both benefits and problems. There should be much greater concern for untoward problems from organisms imported to the U.S., than for indigenous organisms with several new genes added to them.

The major concerns about released recombinant microorganisms seem to be the chance of developing a new disease organism or creating a new organism that will disrupt the environment. If there is no scientific basis to predict the kind of disease or even the host of the disease, then it will be impossible to develop relevant laboratory tests to demonstrate disease-forming properties of the genetically engineered microorganism. Environmental disruption (e.g. water eutrophication) is not caused primarily by contamination with microbes since every environment is potentially exposed to each type of microorganism. Microbes readily travel long distances. Problems occur when there is new selective pressure on an environment. For example, fertilizer runoff into our lakes selects for populations of microorganisms that would not dominate without

the runoff. Therefore, it seems to be an impossible task to demonstrate, by laboratory tests, that a recombinant microorganism (or any other altered microorganism) will disrupt the environment if one is unable to predict, and duplicate in the laboratory, the selective pressures that a recombinant cell will encounter.

The fact that a recombinant microorganism may survive in the soil for many years and may exchange its foreign DNA with other microorganisms should not be a concern. Bacteria considered harmless exchange genes naturally and frequently with pathogens; however, these safe species do not appear as pathogens. If there is selective pressure for increased growth of a dangerous microbe, then even a single cell can do its "damage." If there is no selective pressure to allow the recombinant microorganism to maintain high populations, then trillions of cells added to the environment would not cause a significant problem. No laboratory test can give adequate confidence that a single cell will not remain after a specified time in an acre of field. Thus, if there is realistic concern of danger from a release experiment (whether or not the microorganism is engineered), laboratory data on survival or gene exchange cannot significantly alleviate this concern. Genetically engineered microorganisms, considered through arguments discussed above, will not indicate realistic concerns.

PUBLIC CONCERN

Over a decade ago, when genetic engineering experiments began, the scientific and non-scientific public raised many concerns. These concerns were addressed in public discussions and debate and ultimately the concerns subsided. Almost all recombinant experiments are now performed with minimum containment which certainly causes the researchers involved to come in contact with genetically engineered organisms. Even though hundreds of laboratories around the world are actively engaged in these experiments, not a single disease or environmental problem has resulted from this work. Now that scientists want to purposely add recombinant organisms to the environment to benefit agriculture, we see another series of concerns. These need to be discussed, put into perspective, and regulated on the basis of relevant experience. It is important that imagined scenarios do not unnecessarily precipitate fear and over-regulation of this technology. Genetic engineering has tremendous potential to increase quality of life and to displace practices that now have undesirable consequences.

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Chapter 25

Review of the Safety of Agricultural Recombinant DNA Experiments and Field Tests

The Role of the National Institutes of Health

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In response to a request by scientists, the National Institutes of Health (NIH) established the NIH Recombinant DNA Advisory Committee (RAC) and the NIH Guidelines for Research Involving Recombinant DNA Molecules; these Guidelines, first issued in 1976, and revised many times since then, are safety standards for recombinant DNA work, including agricultural experiments and field tests.

Soon after the first recombinant DNA experiments were performed, both the promise and the possible hazards of such experiments were discussed at a 1973 Gordon Conference. Those present voted that a letter be sent to the National Academy of Sciences and be published (1), suggesting that the academy "consider this problem and recommend specific actions or guidelines."

In response to this initiative, the academy formed a committee of distinguished scientists, chaired by Dr. Paul Berg of Stanford University. These scientists prepared a letter (2) that appeared simultaneously in *Science*, *Nature*, and the *Proceedings of the National Academy of Sciences*.

The letter proposed that "until the potential hazards of such recombinant DNA molecules have been better evaluated or until adequate methods are developed for preventing their spread, scientists throughout the world join with the members of this committee in voluntarily deferring [certain] experiments." This request by scientists for a voluntary "moratorium" on such work while questions of safety were further evaluated was widely hailed in the press.

The letter also proposed that the National Institutes of Health (NIH) establish an advisory committee to write "guidelines to be followed by investigators working with potentially hazardous recombinant DNA molecules."

The letter also called for an international conference of scientists, which was held in February 1975 at the Asilomar Conference Center in California. There were 150 attendees from 15

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countries, plus members of the press who gave the meeting wide coverage. Two journalists subsequently wrote books on the conference (3-4). The final conference report (5) recommended proceeding with most recombinant DNA experiments, using appropriate "physical containment" and "biological containment" (discussed below).

The first meeting of the NIH Recombinant DNA Advisory Committee (RAC) -- formed in response to the letter of Dr. Berg and his associates -- was held the day after the Asilomar conference. (Minutes of all RAC meetings are available from the Office of Recombinant DNA Activities, NIH, Bldg. 31, Rm. 3B10, Bethesda, MD 20892.) After a series of meetings, the RAC, in December 1975, adopted proposed guidelines for recombinant DNA research. When the NIH Director at the time, Dr. Donald Fredrickson, received the RAC proposal, he called a meeting of his Director's Advisory Committee, to which he invited many distinguished scientific and public representatives. (The full transcript of this February 1976 meeting, and all letters of comment on the proposed guidelines, form the bulk of Volume One of what is now a seven volume massive public record (6-12) of the history of NIH Guidelines. The first five volumes in this series can be purchased from the Superintendent of Documents, U.S. Government Printing Office, Washington, D.C. 20402, or viewed in some 600 public libraries of the GPO depository system. Volumes 6 and 7 are available from the Office of Recombinant DNA Activities at NIH.) Following revision based on the suggestions received at the February 1976 meeting and afterwards, the original NIH Guidelines for Research Involving Recombinant DNA Molecules were issued in July 1976 (13).

Although most members of the RAC were, and continue to be, biomedical scientists, there has also been representation by agricultural scientists. Beginning with the third RAC meeting in July, 1975, Dr. Peter Day, at the time with the Connecticut Agricultural Experiment Station, served as an ad hoc consultant, until his formal appointment to the Committee in 1976. Other agricultural scientists who have served as RAC members over the years include: Dr. Milton Zaitlin, Professor of Plant Pathology, Cornell University; Dr. Francis Broadbent, Professor of Soil Microbiology, University of California, Davis; Dr. Winston Brill, University of Wisconsin; Dr. Nina Fedoroff, Carnegie Institution of Washington; Dr. Thomas Pirone, Professor of Plant Pathology, University of Kentucky; and Dr. Anne Vidaver, Professor of Plant Pathology, University of Nebraska.

Original NIH Guidelines

The original 1976 NIH Guidelines included a list of types of experiments that were "not to be initiated at the present time."

The Guidelines then described in detail certain laboratory practices and equipment that defined four levels of "physical containment" called P1, P2, P3, and P4. (These have been modified slightly in the current Guidelines and now are are called Biosafety Levels 1 to 4 (BL1 to BL4).)

In addition to physical containment, a major advance resulting from the Asilomar conference was the concept of biological

containment -- the use, in experiments, of micro-organisms with limited ability to survive outside the laboratory. Most recombinant DNA experiments have been done with the harmless bacterium, *Escherichia coli*, strain K-12. Its use, together with that of certain specified plasmids or bacteriophages constitutes what is called the EK1 level of biological containment. By further modifying *E. coli* K-12 to render the bacteria much less likely to survive, were they to escape from the laboratory (for example, by making them dependent for survival on certain nutrients that are supplied in the laboratory but that do not occur in significant concentrations in nature, and by making the modified bacteria sensitive to sunlight and to bile acids), and by requiring data on survivability to be submitted to NIH and reviewed by the RAC, one arrived at what were called the EK2 and EK3 levels of biological containment.

Having defined four levels of physical containment and three levels of biological containment, the Guidelines then went on to specify levels of physical and biological containment required for each of many different kinds of experiments. There were specific sections dealing with the use of plant DNA and plant viral DNA inserted into *E. coli* K-12, and the insertion of foreign DNA into plants, plant parts, seedlings or plant cells in tissue culture, all to be contained within the research laboratory.

Finally, the Guidelines discussed the roles and responsibilities of the scientist, his or her university, the university's institutional biosafety committee (which in most cases already existed to oversee other potential hazards), and the NIH.

After their promulgation in 1976, the NIH Guidelines were adopted by other Federal agencies including the Department of Agriculture.

In 1977, sixteen different bills dealing with recombinant DNA were introduced in the U.S. Congress, many of which would have made the NIH Guidelines mandatory for private industry. There was great disagreement on a number of provision of these proposed bills, and none ever reached the floor of the full House or Senate for a vote. In the absence of national legislation a number of cities, including Cambridge, Waltham and Amherst, Massachusetts, and Berkeley and Emeryville, California, have mandated compliance with the NIH Guidelines by local ordinance.

Guideline Revision

The first revision of the NIH Guidelines was issued in December 1978 (14). Some of the major changes in the December 1978 Guidelines, as compared with the original, were:

1. In general, experiments were assigned lower levels of required containment.
2. Certain classes of experiments deemed of the lowest potential hazard were exempted entirely from the Guidelines.
3. Increased representation was mandated on local institutional biosafety committees (which oversee recombinant DNA research at individual institutions) and on the RAC.
4. Procedures were built into the Guidelines for changing them in the future.

The RAC had originally been a 14 member committee composed entirely of scientists. At the RAC's own suggestion, a professor of government and a bioethicist were added to the committee in 1976. At the time of the 1978 Guidelines revision, the RAC was expanded to 25 voting members, with the requirement that at least 6 members "be persons knowledgeable in applicable law, standards of professional conduct and practice, public attitudes, the environment, public health, occupational health, or related fields." Also, scientists representing many different backgrounds were added as members, and all relevant Federal agencies were given a non-voting representation on the RAC. The U.S. Department of Agriculture representative, since February, 1979, has been Dr. Sue Tolin.

Perhaps the major change in the December 1978 Guidelines was that a process was built into them for further change. Anyone wishing to suggest a revision of the Guidelines may submit it to NIH. It is then published in the Federal Register, at least 30 days before a regular meeting of the RAC, for public comment. The suggested revision and all written comments received are considered by the RAC at an open meeting; members of the public wishing to speak on the subject are given the opportunity to do so. Following the discussion, the RAC votes on whether or not to recommend the revision. After the meeting, the NIH Director promulgates his final decision on the RAC recommendations in the Federal Register. In this fashion, the Guidelines have been incrementally modified every few months since December 1978 (15-35). (Copies of all these revisions and of any future ones, can be obtained from the NIH Office of Recombinant DNA Activities.)

The Guidelines continue to be mandatory for institutions receiving NIH funding for recombinant DNA research. In addition the Guidelines encourage other institutions (such as those in industry) to comply voluntarily.

The Guidelines state that certain classes of experiments (including "deliberate release into the environment" of an organism containing recombinant DNA) cannot proceed until they are reviewed by the RAC and approved by NIH. In this category, NIH, in June 1983, approved a proposal by Dr. Steven Lindow and Nickolas Panopoulos of the Unversity of California, Berkeley, to field test ice-nucleation-minus bacteria prepared by recombinant DNA techniques for purposes of biological control of frost damage to plants. Before the field test actually began a lawsuit was filed in September 1983 by Foundation on Economic Trends (Jeremy Rifkin, President), et al., charging that the NIH approval violated the National Environmental Policy Act. In May 1984, Judge John Sirica issued a preliminary injunction forbidding the experiment from proceeding. In February 1985, the Court of Appeals partially affirmed and partially reversed the injunction. The case is still ongoing.

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Chapter 26

Report of the Interagency Biotechnology Working Group

Description of the Toxic Substances Control Act

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A biotechnology working group of the Cabinet Council on Natural Resources and the Environment recently proposed a coordinated federal approach to the regulation of new commercial products of biotechnology. This paper summarizes the recent activities of the working group and describes in more detail the proposed approach of the Environemtnal Protection Agency (EPA) particularly under the Toxic Substances Control Act (TSCA). Under TSCA, EPA will have responsibility for reviewing new genetically engineered microorganisms in a range of environmental and agricultural applications, including pollution control and nitrogen fixation. (Foods, pesticides, and drugs are excluded from coverage under TSCA, although EPA will review genetically engineered pesticides under the Federal Insecticide, Fungicide and Rodenticide Act.) This paper discusses EPA's review authority under TSCA, the nature and scope of the TSCA biotechnology program, and the review procedures and assessment principles EPA is now developing.

Since the 1970's, the National Institutes of Health Recombinant DNA Advisory Committee (NIH RAC) has provided oversight for biotechnology research, especially recombinant DNA research. However, it is now clear that the various regulatory agencies concerned with public health and the environment must define their roles in the regulation of the commercial uses of this rapidly growing technology. Toward this end, last year the U.S. Cabinet Council on Natural Resources and Environment formed a biotechnology working group. This group is chaired by the Deputy Director of the Office of Science and Technology Policy and includes representatives from the U.S. Department of Agriculture (USDA), the Department of Health and Human Services (HHS), the Environmental Protection Agency (EPA),

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and several other agencies. The main accomplishment of this group to date has been the preparation and publication on December 31, 1984 of a FEDERAL REGISTER Notice on biotechnology, which presented (1) the proposed policies of the USDA, the Food and Drug Administration (FDA), and EPA toward the products of biotechnology, (2) a regulatory matrix of Federal authorities over biotechnology, and (3) a proposal for a coordinated Federal scientific advisory mechanism for biotechnology. This notice represents a major initial success in interagency cooperation that can provide industry with coordinated regulatory information.

Our presentation today addresses the potential role of EPA's Toxic Substances Control Act (TSCA) within the framework of Federal oversight of biotechnology, as defined by the Cabinet Council working group. Despite the benefits that can be derived from this technology, concerns have been raised about the possible risks involved. These concerns include the possibility that biotechnology products such as genetically engineered microorganisms may have increased virulence, a broadened host range, and/or the ability to replicate and spread beyond the application site. The novel genetic traits induced in these organisms may be transferred to others and microorganisms can be difficult to identify and monitor after their release into the environment.

There are several regulatory issues that are of immediate concern when considering the application of TSCA to biotechnology. I have included in today's presentation three of these issues: first, the distinction between a "new" and a "naturally occurring" chemical substances for the purpose of the applicability of the premanufacture notification (PMN) requirement of TSCA; second, the scope of the research and development exemption from the PMN requirements, and its implication for open field testing of microorganisms; and third the type and amount of information that EPA might need for risk/benefit analyses of new microorganisms. I will also briefly mention some technical areas in which further research would greatly facilitate the processes of risk/benefit assessment.

TSCA

The Toxic Substances Control Act (TSCA) was enacted in 1976 to provide EPA with authority to address risks posed by a broad range of "chemical substances." Under TSCA, EPA can assess and control exposure to these substances through all phases of their commercial life cycle, including research and development, commercial production, use, and disposal.

TSCA coverage extends to chemical substances and mixtures used in a wide range of industrial, commercial and consumer applications. As defined in section 3(2) of the Act a "chemical substance" is "any organic or inorganic substance of a particular molecular identity, including...any combination of such substances occurring in whole or in part as a result of a chemical reaction or occurring in nature..."

When examining the products of biotechnology, such as recombinant DNA and RNA, for example, it can be seen that they are organic chemical substances of particular molecular identities and

therefore TSCA "chemical substances." By extension, a life form is composed of "chemical substances" as defined in TSCA. Additionally, life forms are themselves "chemical substances" under TSCA because they are combinations of organic substances of particular molecular identities that occur in nature or that occur in whole or in part as a result of chemical reactions.

As noted, TSCA coverage extends to chemical substances and mixtures used in a broad range of industrial, commercial and consumer applications. Some examples of the types of microbial products that would be subject to TSCA are microorganisms used to produce pesticides and other commercial chemicals, to convert biomass to energy, for pollutant degradation and enhanced oil recovery, in metal extraction and concentration, and in certain non-food agriculture applications, such as nitrogen fixation. Certain substances are specifically excluded from TSCA, namely, pesticides, tobacco and tobacco products, nuclear materials, foods, food additives, drugs and cosmetics. Even though TSCA does not cover pesticides (they are covered by EPA under the Federal Insecticide, Fungicide, and Rodenticide Act-FIFRA) it does cover the microorganisms that produce pesticides and the intermediates used to prepare pesticides. However, the intermediates used to prepare other substances such as foods, food additives, drugs and cosmetics have not been regulated under TSCA. In addition, while EPA has asserted that microorganisms are covered under TSCA, the December policy statement did not propose that genetically engineered living plants and animals either as whole organisms or as *in vitro* cultures be made subject to TSCA requirements.

Regulatory Issues

Premanufacture Notification Requirements

For biotechnology, the most important provision of TSCA is the section 5(a) requirement that companies notify EPA at least 90 days before beginning to manufacture or import a "new chemical substance" for commercial purposes. This reporting requirement is known as premanufacture notification or PMN. Any substance that is not listed by name on EPA's Chemical Substances Inventory or that is not "naturally occurring" is defined as "new" and therefore subject to PMN requirements.

Because genetically engineered microorganisms and nucleic acids are neither "naturally occurring" nor listed in the TSCA Inventory, they are subject to PMN requirements under TSCA. However, what is "new" or "naturally occurring" is not always clear cut and needs additional definition when applied to this new technology.

In establishing the original Chemical Substances Inventory and the PMN regulations EPA adopted the policy that human intervention at a relatively simple level does not remove a substance from the category of naturally occurring. Under these regulations, the act of isolating a substance from nature by manual, mechanical or gravitational means, or by dissolution in water, flotation, or heating solely to remove water, does not alter its status as "naturally occurring" and make it subject to PMN requirements. On the other hand, chemical substances that are chemically extracted from a

natural substance are not naturally occurring and must be reported under PMN requirements if they are not already listed on the TSCA inventory.

The Agency proposed in the December 31, 1984 FEDERAL REGISTER notice that similar logic could be followed to determine whether a microorganism is new. Naturally occurring microorganisms would be those that exist as a result of natural events or processes or have been developed as a result of limited manipulation of natural processes. For example, the normal events of reproduction or evolution would not create "new chemical substances." The Agency then proposed that the techniques of R-NDA, R-RNA and cell fusion, which allow the combination of genetic material from organisms that do not exchange genetic material in nature, produce organisms that could be considered "new" and subject to PMN requirements. Comments received by EPA in response to the December Federal Register Notice were highly critical of this "process-based" approach, claiming that it would penalize some technologies and was unrelated to any risks that might be presented by the products of these technologies. EPA is working toward resolution of this difficult issue. Basic options for a final policy statement that are being evaluated include:

1. attempting to establish a list of organisms that exchange DNA in nature,
2. developing a taxonomic approach to what is new, and
3. continuing with the criticized process-based approach to what is new.

Small-Scale Field Testing

New chemical substances produced only in small quantities solely for research and development are exempt from PMN requirements. This exemption generally excludes small scale field testing of agriculture chemicals from PMN requirements. Field testing of traditional chemicals has a low risk because the area of application is geographically circumscribed and the small quantity used for testing is not likely to spread away from this site.

Many people have expressed concern that significant risks could occur if research and development open field-testing of new microorganisms was performed without prior Agency review. Unlike traditional chemicals, living, reproducing microorganisms can increase in number and be disseminated from the application site after release into the environment. EPA review at a later, commercial stage, for these types of products may be too late to prevent widespread exposure. Such early review at the research and development stage now takes place under RAC's guidelines and in EPA's pesticide program. Therefore, EPA is considering whether to initiate a policy of reviewing TSCA microbial products prior to field testing.

Information Requirements in PMN Submissions

At present, there is an absence of generally accepted principles of risk assessment for microbial products, especially when their use involves direct release into the environment. For this reason, and

because there is the potential to produce a wide variety of microorganisms for very different applications, EPA believes that for the present much of the information required under TSCA for the evaluation of these products should be decided on a case-by-case basis.

In all cases, certain minimal up-front information would be required to identify the microorganism unambiguously, both to support risk assessment and to list the organism on the Inventory. This would include:

1. the source of the introduced nucleic acids
2. the processes by which the nucleic acids were manipulated including hosts, vectors, etc.
3. identification of the protein or special function produced.

Also required upon submission of a PMN would be information on intended use, the amounts manufactured, amounts released, and disposal methods. However, the amount and type of data required for the risk assessment of a microorganism would vary according to the risk potential of the organism.

This approach is easily accommodated by the specific provisions of TSCA. Little up-front information is generally required for a PMN; no specific battery of testing is mandated. Rather, in an appropriate case EPA has the authority under section 5(e) of TSCA to require by administrative order specific data needed to complete its risk assessment and to prevent commercial manufacture until this data is provided.

For example, the Agency would expect more definitive information for genetically engineered organisms that are intended to be released into the open environment, and/or are able to survive and reproduce in the environment. These organisms would have a greater potential for widespread exposure than those used in a closed system or those that could not survive. Organisms that are derived from or are themselves toxic, pathogenic or ecologically disruptive could also be considered a higher risk and therefore, require more detailed information for risk assessments than organisms that do not have such properties.

In the case of organisms that are released into the environment, information on application techniques and descriptions of the target environments may be necessary to determine the organisms' ability to survive, reproduce, be transported, or exchange genetic material with other organisms. Factors that limit or enhance the mobility or survivability of organisms or their genetic material may also be significant considerations in risk evaluations.

If the organism is used to produce commercial substances, companies may be asked to submit data on the purity of the final product and the presence of any residual organisms or contaminants in the product.

As the Agency gains experience in the review of genetically engineered microorganisms and their products and is able to develop appropriate risk assessment methods, it may be possible to establish more specific guidelines on the levels of test data and other information that might be submitted and how these data might be used in the evaluation of the products of biotechnology.

In Biotechnology in Agricultural Chemistry; LeBaron, H., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

EPA has a strong tradition in the PMN program of establishing a working relationship with PMN submitters to identify data needs prior to submission, and thus encourages manufacturers to consult with the Agency prior to submitting a PMN on a new microorganism. Such prenotice consultations could prove to be an extremely valuable way to expedite the review process. During such meetings, manufacturers would have the opportunity to describe the specifics of their situation and EPA would be able to provide guidance on the appropriate levels of information needed. This approach minimizes the use of EPA's administrative order authority and avoids needless delays in commercial production.

Research Needed

In addition to the policy issues that I have mentioned today there are numerous research areas that need further development to allow for more informed regulatory review. Significant work in these areas is being funded from our laboratories at Gulf Breeze, Florida and Corvallis, Oregon. Of particular interest to EPA are refinement of microcosm procedures to assess survivability and growth of test microorganisms, development of methods to assess the stability of novel genetic traits, and development of genetic markers in engineered organisms to permit identification and monitoring. The Agency is also interested in development of general methods for the assessment of potential adverse effects, and methods to assess transport possibilities.

EPA is devoting substantial resources toward finding solutions to these problems. In 1986, 4.2 million dollars will be allocated to fund extramural and intramural biotechnology research programs.

Conclusion

Currently, we are evaluating the public comments that we received on the December 31 FEDERAL REGISTER notice. We are using these and information compiled from panels of scientific and technical experts as a basis for the formulation of a revised statement of policy on biotechnology. I've hinted today at some of the directions being considered for the key issues of what is "new" and therefore what products are subject to premanufacture notification.

The potential benefits of biotechnology for the U.S. economy are enormous. A wide array of new commercial products, such as drugs, pesticides and other agricultural products, pollution control products, and foods, are expected to be introduced into the marketplace in the near future. New biotechnological techniques, such as recombinant-DNA have been safely used in the laboratory under the oversight of the NIH/RAC.

We believe, however, that the risks of these techniques become increasingly uncertain as we move from the relatively well contained setting of laboratory scientific research into situations in which the products of biotechnology will have full contact with society and the environment. In view of the current uncertainty, EPA, other Federal agencies, and many in the scientific, industrial and public communities believe that some regulatory oversight is appropriate. However, a major question is how much oversight is appropriate. We

must be sensitive to the fact that ill-considered, duplicative or excessive regulation of the new products of this technology could have a disastrous effect on innovation and could drive research and commercial development overseas. Therefore, it is important to develop a balanced and flexible approach that allows new products to move into the marketplace expeditiously while adequately protecting public health and the environment.

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Chapter 27

Biotechnology Products Related to Agriculture

Overview of Regulatory Decisions at the U.S. Environmental Protection Agency

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New agricultural products are being developed using modern biotechnology. This paper discusses one group of such products, genetically engineered microbial pesticides, and the Environmental Protection Agency's (EPA) regulatory activities which affect these products.

Microbial pesticides have been regulated under FIFRA since 1948. Since that date fifteen naturally occurring microorganisms have been registered, including three varieties of *Bacillus thuringiensis*, for use in agriculture, forestry, mosquito control and homeowner situations (Table I). In formulating its regulatory program for genetically engineered microbial pesticides, the Agency is drawing on this past experience in evaluating microbial pesticides. In the last 18 months, the Agency has reviewed several "biotechnology products" under its interim policy on small scale field testing and experimental use permit (EUP) authority. Some products are now ready to be field tested and pose challenging scientific and regulatory questions which are discussed in this paper.

EPA's REGULATORY RESPONSIBILITY

EPA regulates pesticide products as required by the Federal Insecticide, Fungicide, and Rodenticide Act (FIFRA). The Agency's primary responsibility under FIFRA is to weigh the risks of pesticide use (to humans, nontarget species, and the environment) against the potential benefits. This determination is made through premarket screening and evaluation of each product. EPA may then register those products whose benefits outweigh their risks and whose use will not pose unreasonable adverse effects on human health or the environment.

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Table I. EPA - registered microbial pesticides

Microorganisms	Year Registered	Pest Controlled
Bacteria		
Bacillus popillae	1948	Japanese Beetle Larvae
B. lentimorbus	1948	Japanese Beetle Larvae
B. thuringiensis	1961	Moth Larvae (includes garden uses)
B. t. israliensis	1981	Mosquito Larvae
B. t. aizawai	1981	Wax Moth Larvae
Agrobacterium radiobacter	1979	Crown Gall
Viruses		
Heliothis Nuclear Polyhedrosis Virus (NPV)	1975	Cotton Bollworm, Budworm
Tussock Moth NPV	1976	Douglas Fir Tussock Moth Larvae
Gypsy Moth NPV	1978	Gypsy Moth Larvae
Pine Sawfly NPV	1983	Pine Sawfly Larvae
Fungi		
Hirsitella thompsoni	1981	Mites
Phytophthora palmivora	1981	Citrus Strangler Vine
Colletotrichum gloeosporides	1982	Northern Joint Vetch
Protozoa		
Nosema locustae	1980	Grasshoppers

In Biotechnology in Agricultural Chemistry; LeBaron, H., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

The extent of EPA oversight depends on the degree of pesticide use and environmental exposure. That is, use and exposure generally correlate with the stage of product development. For example, small scale field testing would be expected to result in minimal exposure and risk and therefore warrants less EPA oversight. On the other hand, large scale testing and full commercial use may result in more extensive exposure with a greater likelihood of risk. Therefore, EPA generally extends more comprehensive oversight in the latter situation.

Small-Scale Field Testing. Pesticide developers generally need to conduct field studies in order to gather product performance, use, and other types of data necessary to support the registration of their product. Initially, such testing may be conducted on a very limited basis. The regulations governing field studies[1] give the EPA authority to grant an experimental use permit (EUP) to conduct field tests. The EUP regulations include a generally applicable presumption that experimental use permits will not be required for small scale tests of new pesticides (i.e., tests conducted on 10 acres of land or less and on 1 acre of water or less). However, due to special concerns such as the ability to survive and replicate, potential for genetic transfer and ability to spread independently from the site of application, and the potential lack of natural control mechanisms, the Agency concluded that such a presumption was inappropriate for biotechnology products (i.e., genetically engineered microbial pesticides) as well as nonindigenous but naturally occurring microbial pesticides.

As a result, the Agency issued a statement of interim policy[2] addressing small scale field testing of microbial pesticides in October, 1984. The purpose of this policy is to provide a mechanism for the Agency to evaluate small scale field test proposals for their potential for risks to human health or the environment and to determine whether, based on potential risks of small scale release, EUPs are required before initiation of testing. As set forth in the interim policy, information is requested on the microorganism to be tested; its identity, growth, survival and competitive characteristics; host range and potential effects on nontarget species; how it has been manipulated; information on the parental strains; and information on the design of the proposed field study. The amount of information and data may vary depending upon the specific microorganism under review and the manner in which it is to be tested.

Agency scientists use this information to construct a risk scenario appropriate to the particular microorganism. If the proposed field test poses no foreseeable risks and no additional data are needed, then no EUP is needed before the field test. If significant risk questions or the need for additional data or monitoring is

1 U.S. EPA, 1985. Experimental Use Permits; Title 40, Code of Federal Regulations, Part 172.

2 US.EPA, 1984. Microbial Pesticides; Interim Policy on Small Scale Field Testing, FR 49 (202), 40659.

established, then the Agency may determine that an EUP is needed before the field test is initiated and the applicable EUP requirements must be fulfilled.

Large Scale Field Testing and Registration. An EUP must be obtained before large scale field testing of any pesticide, including microbial products. Data and information requirements for an EUP are a subset of those that apply to products for registration and are set forth in a regulation titled Data Requirements for Pesticide Registration (40 CFR 158)[3]. Permission to conduct field testing is granted under an EUP for a specific time period (e.g., one year) on a test plot suitable for the purpose of the test, but limited in acrage. Therefore, the exposure and potential risks associated with such tests may be correspondingly limited.

All pesticides must be registered before they may be used on a full commercial scale. EPA extends the most comprehensive oversight at this stage of development due to the high potential for human or environmental exposure that may result from products registered for a variety of uses on extensive acreage. Accordingly, the full data requirements and procedures as specified in 40 CFR 158 and 162[4] are applicable. Guidelines for developing the necessary data for an EUP or registration are provided in Subdivision M of the Pesticide Assessment Guidelines.[5] Mechanisms are available to increase or decrease the data requirements as appropriate to the specific situation.

CURRENT STATUS

At present, EPA's Interim Policy on Small Scale Field Testing applies to all genetically engineered or manipulated microbial pesticides. This includes products derived by recombinant DNA (rDNA), recombinant RNA (rRNA), cell fusion, transformation, and conjugation as well as products derived by directed and undirected mutagenesis techniques. The Agency solicited public comment on its Interim Policy when it was published and again when the Agency published its Proposed Policy on the Regulation of Certain Microbial Products[6] in December, 1984 as part of a government-wide policy statement on the regulation of biotechnology. We have carefully considered the numerous comments received from industry, trade associations, public interest groups and academics and are now in the process of revising the Interim Policy in response to these comments and the experience we have gained over the past year in evaluating several small scale testing

3 U.S. EPA, 1985. Data Requirements for Pesticide Registration; Title 40, Code of Federal Regulations, Part 158.

4 U.S EPA, 1985. Regulations for the Enforcement of the Federal Insecticide, Fungicide, and Rodenticide Act; Title 40, Code of Federal Regulations, Part 162.

5 U.S. EPA, 1983. Pesticide Assessment Guidelines, Subdivision M - Biorational Pesticides, National Technical Information Service, Springfield, VA, # PB83-153965.

6 Office of Science and Technology Policy, 1984. Proposal for a Coordinated Framework for Regulation of Biotechnology; Notice FR 49(252), 50882.

proposals. A revised policy on small scale field testing should be available in 1986. In the meantime we will expedite reviews for products likely to pose neglible risk.

As of this writing (Winter, 1985), EPA has received and evaluated several proposals to conduct small scale field testing with genetically altered microorganisms. In three cases, EPA determined that an EUP would be required and in another case, determined that the product was not subject to the Interim Policy. Two EUP applications are currently under review.

The Agency has evaluated and approved EUP applications from the Advanced Genetic Sciences Company (AGS). AGS has obtained EPA permission to conduct small scale field testing to evaluate the frost protection potential of two strains of genetically altered bacteria. The Agency reviewed a very similar proposal from the University of California (Drs. Lindow and Panopoulos) and determined that they too would need to obtain an EUP prior to conducting field tests. Their EUP application has been received and is currently under review. In the third case, EPA evaluated a proposal from the Monsanto Company to conduct a field test to evaluate the insecticidal properties of a soil borne bacterium genetically altered to contain the insect toxin producing gene from *Bacillus thuringiensis*. Here again the Agency determined that an EUP would be required. In response, Monsanto submitted an EUP application containing, among other things, data to enable the Agency to evaluate environmental fate and effects on non-target organisms of the genetically altered microbial pesticide. These data are now under review. Further details concerning these and other related proposals are provided in Table II.

The Agency is establishing significant scientific and policy making precedents in its reviews and decisions for these first applications to field test genetically altered microbial pesticides. Therefore, as described in the following section, EPA has instituted a mechanism to obtain outside peer review of its scientific assessments from other Federal Agencies as well as a panel of independent scientists. The balance of this paper concentrates on EPA's regulatory program for small scale applications of genetically altered microbial pesticides, although we expect that larger scale tests and full registration will require a similar review process.

REVIEW PROCESS FOR SMALL-SCALE FIELD TEST PROPOSALS

EPA has up to 90 days to review each notification of intent to conduct small-scale field testing and to determine whether an EUP is required. The Agency encourages prospective applicants to meet with EPA prior to submission of their notification to discuss their field test and determine what specific data and information would be necessary to evaluate the product.

EPA's review process will include some or all of the elements described in the following paragraphs and depicted in Figure 1. As the Agency builds a baseline of risk assessment data and gains more experience in evaluating these products, certain steps may no longer be necessary. In certain cases, an abbreviated review process may be appropriate (e.g., review of a proposal that is similar to an already reviewed case). Such a determination will be made on a case-by-case basis.

Table II. Summary of microbial pesticide notifications and experimental use permits

Applicant & Type of Submission	Type of Product	Chronology of Events	Major Issues
(1) AGS - Notification	• rDNA deletion product • INA^- Bacteria for frost protection - *Pseudomonas syringae* and *P. fluorescens*	• November 84 - Notification received • January 85 - SAP Subpanel Meeting • February 85 - letter to AGS requesting additional information and an EUP	• Potential adverse effect on plants, insects, weather patterns • Competitiveness of INA^- products and potential to displace INA^+ bacteria
(2) AGS - EUP	• rDNA deletion product • INA^- Bacteria for frost protection - *Pseudomonas syringae* and *P. fluorescens*	• July 85 - EUP applications received • August 85 - Announce receipt in FR • Sept. 85 - Received public comments (E. L. Rogers) • Sept/Oct. 85 - Received SAP & inter-Agency review comments • November 85 - announced approval • November 85 - Agency sued by Foundation on Economic Trends	• AGS submitted documentation of their findings, as requested • Additional data submitted on competitiveness, plant pathogenicity and colonization, identification, and detection • Assertions of low risk substantiated by data
(3) U. California Notification	• rDNA deletion product • INA^- Bacteria for frost protection - *Pseudomonas syringae*	• December 84 - Notification received • February 85 - SAP provided comments • March 85 - letter to Lindow requesting additional data and an EUP • June 85 - OPP met with NIH and Cabinet Council (Cohrssen) to discuss the two Agencies' reviews and conclusions concerning INA^- bacteria	• Potential adverse effects on plants, insects, weather patterns • Competitiveness of INA^- products and potential to displace INA^+ bacteria

Continued on next page

Table II - Continued

Applicant & Type of Submission	Type of Product	Chronology of Events	Major Issues
(4) U. California EUP	• rDNA deletion product • INA - Bacteria for frost protection - *Pseudomonas syringae* (2 strains)	• December 85 - applications received • Currently undergoing in-house review	• Agency reviewing data generated by applicant to address major scientific issues identified in notification
(5) Mycogen - Notification	• rDNA addition product • B.T. toxin gene inserted into *Pseudomonas* for insect control; CELLS KILLED	• April 85 - Notification received • June 85 - Mycogen informed that Interim Policy does not apply to their "killed" product.	• Applicability of Interim Policy - Mycogen provided data to substantiate non-viability of their product
(6) USDA - Notification	• Undirected mutagenesis product • Two species of fungi to be used to control pest fungi • *Trichoderma viride* and *T. harzianum*	• January 85 - Notification received • April 85 - 90-day clock suspended; OPP requested additional information • May 85 - USDA withdrew notification	• Relative level of risk posed by products of undirected mutagenesis compared to other genetically altered products • The extent of data and information needed (and is practical to obtain) on these products for notification
(7) Monsanto - Notification	• rDNA addition product • B.T. toxin gene inserted into *P. fluorescens* for insect control	• January 85 - Notification received • March 85 - SAP subpanel meeting • April 85 - Letter to Monsanto requesting additional information and an EUP	• Toxin gene insertion mechanism may cause environmental problem • Possibility of adverse effects on non-target species
(8) Monsanto - EUP	• rDNA addition product • B.T. toxin gene inserted into *P. fluorescens* for insect control	• October 85 - EUP application received • Currently undergoing in-house review • Will be reviewed by the SAP subpanel and other agencies	• Agency reviewing data generated by company to address major scientific issues identified in the notification • Determination of Confidential Business Information status of the submission

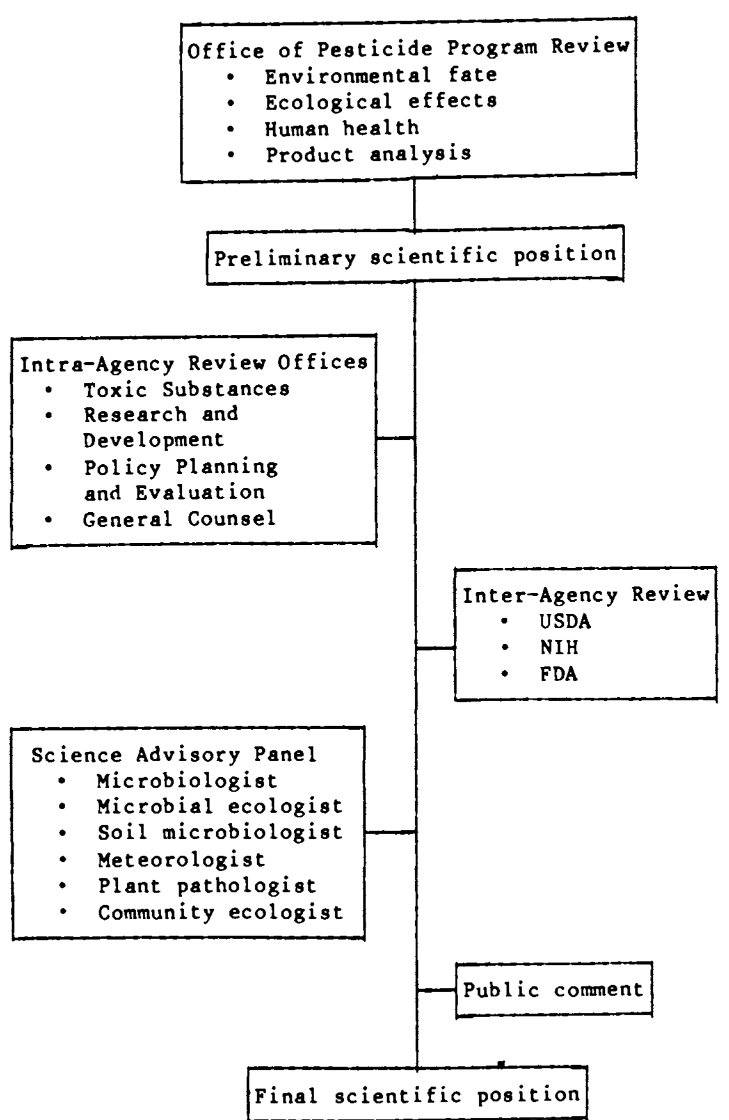

Figure 1. EPA assessment process.

Once a notification is received, scientists in the Office of Pesticide Programs (OPP) review each proposal and assess the potential risks associated with the proposed experiment. OPP reviewers are trained in the relevant disciplines, including microbiology, microbial ecology, and molecular biology. As noted above, a risk scenario is constructed for each proposed test using the data and information submitted by the applicant. OPP develops a written scientific position for each proposal which identifies potential problems or significant unanswered questions and sets forth a statement of the overall likelihood of significant risk from the proposed field test.

OPP obtains comments on its assessment from a workgroup within EPA and from several other federal agencies (USDA, National Institutes of Health, Food and Drug Administration, and National Science Foundation). Their comments are incorporated into the scientific position, as appropriate.

OPP contacts the appropriate state pesticide regulatory authorities to ensure that they are aware of the proposal and to discuss EPA's assessment. These contacts ensure that the actions of EPA and the state agencies are as consistent as possible. OPP also notifies the Animal and Plant Health Inspection Service (APHIS) of the USDA so that they can determine whether any aspect of the proposed experiment falls within APHIS jurisdiction and, if so, to avoid duplicative or conflicting assessments.

If the notification raises complex or controversial scientific questions, OPP provides the notification package and its scientific position to a group of independent scientists constituted as a subpanel of OPP's Scientific Advisory Panel (SAP). Separate subpanels may be formed to review each proposal since each microorganism and its proposed use may differ and raise questions that require the analysis of individuals with different expertise. The purpose of the SAP subpanel is to obtain an independent peer review of the OPP scientific position, to address specific scientific questions raised by OPP, and to identify any additional points, questions or problems.

At the conclusion of the review, the Agency then decides whether an EUP is required. The decision document sets forth OPP's conclusions with respect to potential risks associated with the proposal, identifies any remaining questions or additional data that may be needed to complete the risk assessment, and, if an EUP is required, may recommend restrictions, limitations, or modifications of the proposal to address areas of concern. If an EUP is not required, the applicant may proceed with the proposed field test. If an EUP is required, the applicant must apply for a permit, providing the necessary data and information required to support the application. The Agency may decide to require an EUP to ensure that the experiment is conducted with certain defined limits, or in order to obtain the necessary data to assess the proposal, or to ensure that certain kinds of data will be developed during the test and reported to the Agency.

RISK FACTORS

EPA has identified several factors that may contribute to risks from the environmental application of genetically altered microbial pesticides. EPA has considered these factors in developing its

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policies concerning these products and in specific field testing proposals. It should be emphasized that the Agency believes these are important to consider in the risk assessment, but that they do not necessarily constitute a risk in any specific situation.

Pesticidal Nature. The products discussed in this paper are pesticides and by their nature are designed or intended to have some biological activity to "control" other populations of organisms (i.e., pests). For example, microbial pesticides may control pests by infective/pathogenic action, production of a toxin, by affecting a pest's growth or reproduction, or by physically displacing a pest organism from its normal ecological niche. As with chemicals, the concern is that nontarget organisms may be adversely affected as well.

Environmental Application. Many pesticides are applied outdoors to major food crops, forests, and rangeland. These applications may encompass vast acreage using aerial or ground broadcast sprays. This is in marked contrast to other uses of genetically altered microorganisms in fermentors, laboratory or other physically confined situations. Therefore, there maybe significant potential for human and environmental exposure to genetically altered microbial pesticides when used on a full commercial basis.

Competitiveness. Unlike chemical pesticides, microbial pesticides are living entities which may survive, replicate and compete with other species for space and food sources. These factors, alone, do not constitute a hazard, but do require an evaluation for which there is no counterpart for chemical pesticides. Such an analysis must consider whether characteristics have been engineered into a microorganism which may permit it to establish itself in new environmental niches. If so, then its potential to exert adverse effects on nontarget species must be considered.

Ecological Effects. Classical chemical toxicity testing, alone, is inappropriate for most microbial pesticides. Toxin production is one aspect to be considered. However, infectivity and pathogenicity must also be evaluated as potential end points. Host range is also a relevant question for microbial pesticides that may have pathogenic or toxic effects on their target hosts. For example, if an organism is engineered to produce a new toxin, then we must evaluate whether or not the host range will be extended beyond the pest species.

Moveable Genetic Elements. It is known that genetic material can be transferred among organisms in nature. However, the significance of this phenomenon and the extent to which it may occur in the environment is not understood. Depending on how an organism is engineered, genetic material may be subject to movement to other species and this may be of concern if the transferred genetic material enables recipient organisms to elicit adverse effects.

Human Health. The likelihood of risks to human health is very low for genetically altered microbial pesticides used in small scale field studies. This assumes that the parent microorganisms is not a mammalian pathogen and that we have a good understanding of the parental strains and how they have been manipulated. Larger scale applications may involve more human exposure (e.g., to applicators) and would warrant more extensive examination, particularly with respect to potential for allergenic effects. In any event, the Agency believes that full handling precautions and protective clothing should be used as a prudent and practical measure even in small scale field tests. Finally, the use of antibiotic resistance markers is not of great concern provided the markers used are not for clinically important antibiotics. If used wisely, the benefits of using antibiotic resistance markers for detecting and isolating the microorganism in the environment would appear to outweigh the potential risks.

Thus far, OPP's reviews of small-scale field testing proposals for genetically altered microbial pesticides have addressed several of these risk factors. For example, OPP has identified potential risks associated with the transfer of inserted genetic material to other organisms, the competitiveness of the engineered organism compared with the parental organisms in the environment, and the ability of the engineered organism to become established in a new ecological niche and thereby pose a potential adverse enviromental impact.

OPP has addressed these and similar questions on a case-by-case basis in its risk assessments. To answer these questions, EPA has normative data requirements and test methods developed for naturally occurring microorganisms. Use of these tests (or tiered series of tests) enable the Agency to to establish whether the effect of concern is likely to materialize under field conditions. In some cases, applicants have addressed questions by revising the conditions of the proposed test or by modifying the test microorganism to minimize potential risk.

UNANSWERED QUESTIONS

During the course of EPA's review of field testing proposals, significant questions of science policy have been identified. Following are some examples:

Review of "Similar" Microorganisms. To what extent can data developed on one strain of a species be used to assess a closely related strain (e.g., one that has been subjected to further "minor" genetic alteration)? How different must two strains be to warrant a complete data set on each strain? To the extent possible, EPA would wish to avoid duplicating complete data sets on similar strains or minor genetic modifications.

Test Protocols. What are the most appropriate test species for assessing risk to nontarget organisms. How adequate are tests on surrogate mammalian species for assessing human health effects? Can

microcosm or multiple species testing be used to provide a better predictive measure of adverse effects of microorganisms and how useful are the results obtained from growth chambers or contained greenhouses in predicting fate or effects under actual field conditions?

Further laboratory and greenhouse test methods development is needed to assess the potential adverse effects of genetically altered microbial pesticides. Although some test methods are available and analysis of field testing proposals can proceed on a case-by-case basis, there is a need for further research in test method development.

Field Testing in Remote Sites. How effective is field testing in remote sites as an approach to containment? Further analysis is needed to determine whether this is a practical (or valid) approach, particularly when balanced against the need to fulfill the applicant's objective for conducting the test - which is usually to obtain data on whether the product will work under field conditions.

Environmental Monitoring. How useful are field monitoring data for assessing potential adverse effects of genetically altered microorganisms applied in the environment? A basic dilemma arises in interpreting the results of any effort to monitor microbial populations in the environment. Due to the inherent limits of detection for any microorganism, its apparent absence does not mean that it is not still present in low, but undetectable numbers. Under proper environmental conditions, the microorganism may reach high numbers and be ecologically significant.

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Chapter 28

Critique of Industry Comments on the U.S. Department of Agriculture Policy Statement on Regulation of Biotechnology

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Application of biotechnology-recombinant DNA/RNA, gene transfer, hybridoma, superovulation, embryo transfer, etc., to agriculture offers significant commercial opportunities, as well as regulatory uncertainties. FDA regulation of biotechnology-derived products, therapeutics, diagnostics, feed, food and color additives, and foods will occur under existing statutes on a case-by-case basis. The potential questions each such product presents to the FDA regulatory scheme will be explored, along with the Agency's ability to respond to them.

USDA regulation of agribiotechnology encompasses both R&D and product issues, imports of cell lines, organisms and vectors, postmortem inspection of genetically engineered animals, therapeutics and diagnostics, food standards, and plants. No specific regulatory restraints are now directed toward biotechnology-derived agricultural products. USDA believes "[t]o date no unique or safety problems have been associated with products of genetic engineering, conventional or modern." Safety issues loom on the horizon and have been brought to the public's attention, therefore must be addressed by industry.

In the next few years, biotechnology-derived chemicals will be applied in every major area of agriculture --

<u>plants</u> - new varieties, growth enhancers, fertilizers, pesticides, rodenticides, fungicides

<u>animals</u> genetic manipulations (new strains), embryo transplants, pharmaceuticals, biologicals diagnostics, growth stimulators, feeds

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foods - additives, processing aids, products, enzymes, supplements, ...

Despite the glowing promises which biotechnology offers agriculture, two major unknowns exist: Are biotechnology-derived chemicals commercially feasible? Are they acceptable to regulators?

Commercial feasibility can now only be measured by the considerable interests and investments that agriculturally-based firms are making in biotechnology, since very few biotechnology-derived agricultural products have been marketed.

The FDA and USDA regulatory issues related to agricultural chemicals and products must be viewed from three perspectives: that of the regulatory agencies, the regulated industry and the public. Each perspective has legitimate concerns that must be adequately addressed and to some extent assuaged.

If regulatory concerns are subordinated to commercial concerns by industry, then a restrictive legislative/public backlash will probably occur, particularly if there are perceived health or environmental risks. Conversely, if there are restrictive regulatory burdens, then the agricultural biotechnology industry will not grow and may move to less restrictive countries. In this situation both the U.S. economy and the public will suffer. Obviously, there must be a balancing of interests and needs.

FDA and USDA policies toward agricultural biotechnology were presented in the December 31, 1984, Federal Register Notice: Proposal for a Coordinated Framework for Regulation of Biotechnology. These agency statements delineate some of the agencies' overt regulatory concerns. The comments submitted in response to this Federal Register Notice evince the concerns of both the industry and the public.

Both regulatory agencies have hidden concerns or agendas that were not articulated in the Federal Register, but which surfaced during negotiations on product submissions. Likewise, industry and the public failed to voice all their concerns.

Summary of USDA Statement of Policy for Regulations Biotechnology Processes and Products

The USDA Policy Statement emphasized the belief that USDA oversight of agricultural biotech was needed at all stages: research, development, testing, evaluation, production, application and disposal. Thus, USDA saw itself as the privotal regulatory agency with requisite statutory authority.1/ Their comment that "To date, no unique or safety problems have been associated with products of genetic engineering, conventional or modern," is based presumably on both commercial development and their own extensive research experience in the Agricultural Research Services (ARS).

The comparison of conventional to modern genetic engineering indicates that USDA views biotechnology as merely an extension of classical mutagenesis, cross-hybridization and other breeding techniques. This understanding and philosophical approach runs throughout the Policy Statement. Based on its own extensive research experience with biotechnology procedures, USDA asserts that such products "... are not fundamentally different from products obtained by conventional technology."

In Biotechnology in Agricultural Chemistry; LeBaron, H., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 1987.

USDA therefore concluded in its Policy Statement that "the existing regulatory framework of USDA combined with NIH Guidelines ... are adequate and appropriate for regulatory research, development, testing and evaluation, production and application of these biotechnology [derived] products."

The Policy Statement reviewed the existing regulatory framework for veterinary biological products, plants and plant products, seeds, and meat and poultry products. Inherent in the USDA review was a safety theme, with the few detailed discussions directed toward the quality and types of data biotechnology-derived products should have. One interesting statement regarding safety was, "The specific cloned nucleotide segment coding for the desired product or other foreign DNA segments must be defined in data supporting each license application." USDA obviously is concerned that non-desirable genes may get transferred into new biotechnology derived strains. In nature, detrimental genes transferred into new strains usually compromise the viability of the new strains.

The review of existing regulations applicable to biotechnology-derived products offered no particular guidance. Obviously, such guidance will come through USDA review on a case-by-case basis.

Several interesting concerns were not expressed overtly, but rather through a statement of jurisdiction. First, the problem of importation of cell-lines, vectors, exotic pathogens, etc., was brushed over. Secondly, USDA asserted that ice nucleation negative bacteria, *Pseudomonas syringae*, are plant pathogens, whether genetically engineered or natural mutants, and thus may be regulated by USDA, in addition to EPA's regulations.

Although USDA's Policy Statement appeared unprovocative, industry raised some interesting concerns and requests for clarifications. The nature of these concerns indicate the current flow or direction of interest in agrobiotechnology.

USDA Issues Perceived by Industry

Of the 32 comments directed toward the USDA Policy Statement, only half were from industry, 11 from trade associations and 5 from political groups or individuals. Of the 16 companies commenting, only 6 were the newer biotechnology-based firms, e.g., Calgene, Agracetus, Biotechnica International, Syntro Corp., Ceva Labs., and Molecular Genetics, while the remainder were from the more established firms in the agricultural, pharmaceutical, chemical, food and plant-breeding areas.

The rather few comments from the estimated 48 biotechnology-based firms claiming agricultural interests have several possible explanations. First, newer biotechnology firms, at this time in their development, lack sufficient regulatory resources or sophistication. Secondly, they may see themselves as primarily research and development oriented and as likely to license out products to larger firms with marketing and regulatory capabilities.

As a corollary to these explanations, I have found that surprisingly few of the newer biotechnology firms -- particularly the smaller ones -- subscribe to the *Federal Register*. In biotechnology, there appears to be greater reliance on trade

associations to represent corporate interests than in traditional industries.

The comments, except those from public interest groups and individuals, supported USDA's policy that its existing statutory and regulatory framework is adequate and no new legislation or regulations are needed. Industry commentors felt that either new administrative procedures or points-to-consider were needed to provide guidance to industry and flexibility to the Agency to regulate a rapidly evolving area. Public interest groups favored new legislation and regulations.

An examination of industry's comments provides a good insight into some of the major concerns that the biotechnology industry has regarding USDA's intended regulatory policies:

No Presumption of a Hazard

Agricultural chemicals, products, foods, etc. derived by biotechnology should not be presumed to be unsafe or hazardous. The final product should be the focal point of USDA regulations, not the biotechnology developmental or manufacturing process. Industry asserts that the regulatory burdens should be identical for identical products irrespective of how they were manufactured. For example, acetone produced by a genetically engineered *Pseudomonas* is identical with acetone produced by organisms derived by conventional mutation or selective media methods.

This argument is also applicable to more complex chemicals, e.g., proteins, lipids and carbohydrates. Obviously, where the chemicals from the genetically engineered organisms are physicochemically identical with those available by conventional processes, no problems should arise. Regulatory uncertainties arise where the genetically engineered products vary in their amino acid composition, side groups, carbon chains, etc. as compared to similar chemicals produced by traditional processes. Industry asserts in its comments that such variations are not meaningful as long as they do not affect the total identity or activity of the complex chemicals. In nature, such variations frequently occur spontaneously and, if not detrimental to viability, are conserved as variants.

Industry is concerned that USDA will perceive any variations in structure, no matter how minor, as constituting new chemical entities, thus requiring full regulatory review and approval.

The same comments were made by industry regarding agricultural organisms produced by genetic engineering in that they also should be regulated the same as organisms produced by traditional microbiological methodology. Again there should be no assumption that they present a hazard. These comments also included recombinant DNA manipulated plants to produce, e.g., disease resistant, chemical resistant, higher yield or environmental/weather resistant varieties. Industry expressed these concerns even though USDA gave assurances in their policy statements that USDA perceived no differences between biotechnology - and traditional microbiological-derived organisms/plants.

These USDA statements did not seem to assuage industry's continuing concerns that there is a hidden USDA regulatory agenda to subject genetically engineered varieties to more stringent requirements. In addition, regulators faced with new technology traditionally tend to be much more conservative and restrictive. Although industry comments supported USDA's proposed case-by-case review which provides for Agency flexibility, industry remains concerned given the traditional propensities of regulators.

Field Testing

The industry comments supported USDA jurisdiction over plants and nonpesticidal microorganisms derived by genetic engineering techniques. Regulation by USDA probably appeared much more attractive, especially given the EPA Policy Statement and the undercurrent which accompanied it. Industry urged that the issue of jurisdiciton between USDA and EPA be resolved.

In addition, commentors wanted USDA to preclude states or local governments from seizing jurisdiction over field testing of genetically engineered plant or organism. Local restrictive regulations coupled with federal indecisiveness would create confusion, thus corporate reluctance to develop such products.

Industry also urged USDA to provide guidance for field testing. One mechanism suggested was for USDA to adopt the informal Points-To-Consider approach, which has been successfully utilized by FDA, Office of Biologics. Interim guidelines arising from agency-industry-public interactions are preferable industry believes to random _ad hoc_ decisions on a case-by-case basis.

It was suggested that USDA define classes of experiments and which classes would be subject to prior notification; organisms presenting no undue risk would not be regulated. USDA should therefore only regulate organisms which present undue risk based on knowledge and/or data. Several review mechanisms were presented by industry: (1) accept or reject the NIH guidelines, (2) increase academic and industry participation on the Agricultural Recombinant DNA Research Committee (ARRC) to make it a more effective review body, and (3) integrate its biotechnology research expertise in the Agricultural Research Service (ARS) into the review process.

Veterinary Biologicals

The regulation of biotechnology-derived veterinary biologicals proposed by USDA raised several major questions. These concerns represented both perceived and historical concerns regarding jurisdiction, as well as the need for explicit guidelines.

There were jurisdictional concerns that an explicit statement is needed that veterinary biologicals are clearly outside the regulatory scope of the Toxic Substance Control Act enforced by EPA. Here again industry perceives EPA's broad policy statements overlapping with USDA. Although not commented on, industry should also feel concern about FDA's jurisdiction. FDA has already defined bovine interferon as an animal drug and not a biological. If interferon, therefore all lymphokines, are animal drugs, can vaccines be far behind?

Several firms commented that any restraints imposed on interstate USDA-licensed animal biologic manufacturers regarding biotechnology processes should also be imposed on intrastate manufacturers. Unlicensed intrastate veterinary biologics manufacturers should not have an economic advantage over licensees. This position was strongly argued by a large animal health industry trade group as well. All the commentors raising these issues were interstate manufacturers regulated by USDA. Intrastate manufacturers pursuant to the holding in Grand Laboratories, Inc. v. Heckler, are regulated by FDA. Given the FDA's proposal regarding food additives which are extremely stringent, this concern seems misplaced.

Industry urged USDA to develop guidelines for veterinary biological products derived by biotechnology processes. Again industry seeks guidance whereas the Agency prefers a case-by-case approach until it has accrued sufficient experience. In one situation, a firm challenged the USDA requirement that the entire nucleotide sequence of foreign DNA cloned into a vector be given. Surprisingly few industry comments raised specific objections to the USDA proposals.

Recombinant Animals

No industry comments were directed toward the area of animal genetic engineering studies. At this juncture, this area was not receiving the high level of commercial activity that plants and bacterial organisms are receiving. It is interesting to note that the Agricultural Research Service (ARS), a division of the USDA, has been a leading researcher in this area.

Importation of Cell Lines

An interesting footnote regarding this Federal Register Notice, concerns by the biotechnology industry comments regarding USDA delays (9-12 months costing $10,000 for animal testing) in processing permits to import cell lines. The Association of Biotechnology Companies (ABC) as well as the Industrial Biotechnology Association (IBA), and several firms raised this concern. Dr. James Glosser, Assistant to the Administrator of APHIS, responded directly to the ABC ad hoc working group repesented by Dr. Brandon Price, Damon Biotech, Dr. Robert Judge, Charles River Biotechnical Services, and Dr. Bruce F. Mackler, ABC General Counsel, who subsequently met with Dr. Bert W. Hawkins, APHIS Administrator and staff to discuss the problem. APHIS in response to the industry instituted significant change decreasing the administrative paperwork so as to condense the time frame to 60-90 days and reduce animal safety testing costs of $2,000 to $3,000. Copies of the APHIS, Veterinary Service Notice are available from ABC, Suite 615, 1220 L Street, N.W., Washington, D.C. 20005.

Perspective on European Patent Situation Regarding Biotechnology

In Europe, great uncertainty exists as to the protectability of plant and animal cell cultures under the European Patent Convention

and their deposit under the Budapest Treaty. Thus far, they have been "shoehorned in" as "microorganisms," reports Iver P. Cooper, Patent Counsel to the Association of Biotechnology Companies. Mr. Cooper met recently with Finnish Patent Office Director General Kivi-Koskinen and Head of Section Hely Lommi, EPO International Relations Administrator Larissa Gruszow and Examiners Bruendl and Galigani. A recent patent application claiming seeds has further perplexed examiners.

The European experts differed sharply as to whether a prior written description of a novel microorganism or plasmid, absent assurance of availability, could be considered part of the State of the art. Typically, the examiners thought that it was while, the lawyers, took the view that it was not prior art.

One European attorney warned Mr. Cooper that the use of the "recognized expert" procedure for inspection of deposits under EPC Rule 28a conflicted with German judicial rulings and might resulting in the unenforceability of the European Patent in Germany.

Mr. Cooper asked several members of the European Bar whether they advised filing national, rather than European applications, in view of the uncertainties as to enforcement of European patents resulting from differences in claim interpretation from country-to-country. They felt that the EPA had by far the greatest understanding of biotechnology, and assured the fairest examination. The German patent examiners, they suggested, would reject genetic engineering inventions on frivolous grounds so as to "pass the buck" to the appellate board.

New European Strict Product Liability Law Affecting Biotech Products

Biotechnology companies already anguishing over U.S. product liability concerns, as well as the lack of available coverage, are now facing strict liability laws in Europe. On July 25, the European Economic Community adopted new liability laws for defective products which member states "shall bring into force, not later than three years from the date of notification of this Directive," that is by July 25, 1988.

Article 6 defines a defective products as one which does not provide the safety which a person is entitled to expect. Thus, the expectation of a "reasonable" purchaser or conversely what a "reasonable" manufacturer will produce for a reasonable purchaser appears to be the standard. The qualifying circumstances in Article 6 include; (1) the presentation of the product, (2) the "reasonably" expected use of the product, and (3) the date of manufacture (state-of-the-art). The presence of better products in commerical distribution does not itself establish that a lesser product is defective.

Article 7 protects the manufacturer from liability if (1) the defect arose when the product was in commercial distribution; (2) the defect is due to compliance with mandatory governmental standards (a defense not available under US law); (3) the state-of-the-art at the time of manufacturing did not enable the existence of the defect to be discovered; and (4) if a component, the defect is a design defect and not attributable to the component.

The Directive containes some of the concepts expressed in the U.S. in the Second Restatement of Torts, Section 402A; but there are also some unique features. Biotechnology companies would be well advised to familiarize themselves with this new law.

ABC General Counsel is preparing a critique on the EEC Directive and will distribute it to its members along with copies of the Directive. Members should call the ABC office to request copies 800-435-9ABC.

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Chapter 29

Risk Assessment and Regulation of Genetically Engineered Products

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The rapid advancement of research in the area of recombinant DNA (rDNA) and the impending commercialization of many genetically engineered products (GEP) have posed a dilemma both to industry and to government regulators. To provide guidelines for adequate safeguards and containment practices, the Recombinant DNA Advisory Committee (RAC) of the National Institutes of Health developed (and later revised) recommendations for the appropriate conduct of experiments involving rDNA. These guidelines were not intended to be regulations, and do not readily apply to the much wider range of commercialized products. Therefore, it is essential that satisfactory regulatory guidelines be developed which not only will support continued development in this promising area, but also will safeguard human health and the environment. If defensible guidelines are to be developed for GEP, it is imperative that there be a clear understanding of the similarities and dissimilarities between "natural" and "unnatural" processes and organisms in the production of GEP. These issues will be discussed as well as the appropriateness of using existing toxicity testing requirements for the risk assessment of GEP.

Biotechnology has "emerged as a global discipline" (1), has been identified as an area which offers significant new opportunities for future industrial growth, and has been designated as a priority for research and development by most governments (2).

National Biotechnology Strategy

In 1980 the Canadian federal government, through the Ministry of State for Science and Technology (MOSST), established a Task Force on Biotechnology to provide advice on effective strategies to promote biotechnology in Canada. The development plan delineated by the Task Force (3) encompassed the following elements: a long-term commitment with allocation of federal monies as a catalyst to

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encourage industrial and provincial government investment; industrial stimulation through tax incentives, direct government assistance, and transfer of biotechnological advances from government and university laboratories to industry; and additional financial support to the National and Medical Research Councils (NRC and MRC) to encourage interdisciplinary research and development and to increase training programs to meet the manpower demands.

The areas of application of biotechnology which were recommended for development in Canada were nitrogen fixation and plant strain development, human and animal health care products, cellulose utilization and waste treatment, and microbial mineral leaching and metal recovery. The Task Force also recommended modifications to the Patent Act to align it more closely with international patent laws, that Bill C-32 Plant Breeders Rights be given careful consideration, that the MRC continue to administer its guidelines for the handling of recombinant DNA, animal viruses and cells (4-7), and that the Department of National Health and Welfare establish a system of voluntary compliance for industry with respect to these guidelines.

In 1983, acting on the recommendations of the Task Force and in support of the National Biotechnology Strategy, the federal government committed funds to strengthen its own research activities and to match funding invested by industry in universities or provincial research organizations. The provincial governments are also promoting research and development through joint ventures with industry, provincial research activities and development companies. Although joint government/industry investments total approximately $300 million, this effort has been criticized as being too small and reflective of a lack of confidence and of entrepreneurial spirit (8). Nevertheless, the federal government is proceeding with its National Biotechnology Strategy, which has four primary objectives: to focus biotechnology research and development on areas of strategic importance to Canada; to ensure an adequate supply of appropriately trained people; to encourage communication amongst participants; and to create a climate conducive to industrial investment in biotechnology. Recent initiatives are also focussing on strengthening the commercial development and marketing components of the strategy in the second phase of the National Biotechnology Strategy.

Implementation of Strategy. The federal biotechnology program is administered through MOSST and the Minister is advised on developments in biotechnology and policies required to promote biotechnology in Canada by the National Biotechnology Advisory Committee, which is comprised of members from industry, federal government and universities.

A Federal Interdepartmental Committee on Biotechnology also has been established to review proposed federal government activities and to monitor the progress of the National Biotechnology Strategy. Biotechnology Networks have been established to enhance collaboration between users and producers in the five strategic areas: plant strain development and nitrogen fixation (N-FIX); human and animal health care products (BIONET); bacterial mineral leaching and metal recovery (BIOMINET); cellulose utilization (BIOFOR); and waste utilization (BIOQUALITY).

One of the current activities of the Interdepartmental Committee is to review the need for legislative changes which pertain to developments in biotechnology and this is being done through the Working Group on Safety and Regulation.

Federal Guidelines and Legislation Relating to Biotechnology

Uses of biotechnology are hardly new, but recently devised techniques have initiated a revolution in genetics with potential genetic manipulation of any living species. The fear that accompanied the birth of modern genetic engineering has led to a more sober and mature concern about reasonable ways to ensure that human health and the environment are protected adequately against unforeseen biotechnological hazards. It is clear that these recent advances in biotechnology have resulted in the public's perception that some form(s) of regulation is required before commercialization occurs.

One of the main issues is whether existing legislation, developed primarily for the control of the chemical industry, is suitable for the regulation of the biotechnology industry with its broad array of new products and organisms that can multiply, mutate, exchange genetic material and migrate. In addition to identifying the specific act that might be used to regulate a product or organism, another issue is to assess whether the data requirements are sufficient to ensure that the regulated products or organisms do not pose an unacceptable risk to human health or the environment.

A further complication in Canada is the shared responsibility of federal and provincial governments for the protection of human health and the environment. It will be essential that all jurisdictions cooperate to develop a rational and effective process for regulating biotechnology.

This paper will focus on the applicability and adequacy of federal legislation for assessing human health hazards from newly created organisms or their products which are used as pest control products or may directly or indirectly find their way into the environment. Brief mention will be made of products which are used as drugs to illustrate regulatory steps that have already been taken by the federal government in the field of biotechnology. The implications of deliberate release of newly created organisms will be discussed primarily in the context of the potential impact on human health.

Guidelines for Recombinant DNA Research. Following the Asilomar Conference on Recombinant DNA Molecules (9), at which safeguards and containment practices were recommended to U.S. officials, an ad hoc committee was struck by the Canadian MRC. The draft guidelines for recombinant DNA (rDNA) research in Canada were issued in 1976, and final Guidelines for Handling of Recombinant DNA Molecules and Animal Viruses and Cells were published in 1977 (4). Analogous to the U.S. National Institutes of Health Recombinant DNA Advisory Committee (RAC), the MRC Guidelines were established to minimize potential risk to human health from research conducted in laboratories funded by MRC. However, the MRC Guidelines are more comprehensive in scope than guidelines developed in other countries by inclusion of animal viruses and cells rather than concentrating only on rDNA. The Biohazards Committee, made up of 4 scientists

and 4 non-scientists with a non-scientist chairman, was established to advise on implementation and revision of the Guidelines, and revised versions were issued in 1979 and 1980 (5-7). In Canada, this process developed with restrained response and without the controversy that occurred in the United States (10).

The MRC Guidelines have been used much more widely by now than originally intended. Current revision will consider perceptions of potential hazard and bring containment levels in line with worldwide standards for use in biomedical research and clinical diagnostic laboratories. This task will be accomplished by a new technical subcommittee, comprised of members from MRC and other relevant sectors of the government, which will report to the MRC Standing Committee on Ethics in Experimentation.

Research using rDNA techniques has advanced rapidly toward commercialization, where the issues are different, particularly with intentional release where more vigorous strains may be developed than were used in the research laboratory. There are external pressures concerning the bioethics of certain developments and the protection both of the environment and of a growing group of industrial workers. Although existing laws regulate chemical processes and products, decisions must be made as to whether biotechnology should be and can be accommodated within these laws.

Review of Present Legislation. In Canada, the primary federal acts that have been developed for the regulation of chemicals and which could potentially be utilized to regulate biotechnology are the Pest Control Products Act (PCP Act), the Food and Drugs Act (F&D Act) and the Environmental Contaminants Act (EC Act).

The PCP Act, which is administered through the Department of Agriculture, and the F&D Act, which is administered through the Department of National Health and Welfare (NHW), require premarket testing and evaluation of products prior to commercialization. Both of these Acts appear to be sufficiently broad to enable regulation of both chemicals and microbes developed through biotechnology.

The Environmental Contaminants Act is administered jointly between the Departments of Environment and NHW, and may be used to control the release, importation, manufacture and processing of a substance and its content in products that the government has reason to believe may pose a threat to human health or the environment. The burden of proof for hazard is on the government and under the existing Act it is difficult to acquire additional information on new chemicals in the absence of sufficient data to demonstrate an environmental or health hazard. Although chemicals which are imported or manufactured in excess of 500 kg are subject to notification requirements under the Act [Sub 4(6)] there is no provision for either premanufacture or premarket notification (PMN), which means that a chemical may already be in the marketplace before concerns are raised about its safety. The current definition of a substance under the Act would preclude its use for regulating microbes. Therefore, it appears that the products of biotechnology that are not used as pesticides or drugs cannot be adequately regulated under existing federal legislation. The EC Act is currently being considered for amendment, and it is possible that this might result in its being suitable for at least some aspects of control for biotechnology products.

The question of which other federal and provincial laws also might be used to regulate biotechnology is under assessment by the Interdepartmental Working Group on Safety and Regulation (IWG). A report is due in December 1985.

In order to assess whether the data requirements under the act are sufficiently broad to ensure safe use of chemical and microbial products, the scope of biotechnology must be delineated and the various components that are to be regulated clearly defined. A working definition which has been used in Canada is that "biotechnology is the application of science and engineering to the direct or indirect use of cells from plants or animals, of micro-organisms, in their natural or modified forms, for the production of goods or the provision of services" (11). A number of techniques are involved, including genetic engineering, enzymes and enzyme systems, fused-cell techniques, plant-cell culture and process and systems engineering. The techniques in turn can be applied to a number of industrial processes, some totally contained and others which are open. Many products are foreseen from the application of the techniques of biotechnology: new plant strains, chemicals, biological control pesticides, pharmaceuticals, substitute fuels, and single cell protein.

Health Hazard Assessment for Chemicals

Not only are diverse processes utilized in biotechnology, but the end products may be inanimate chemicals or actual living organisms. The dilemma is whether these products of modern biotechnology, specifically genetic engineering, are unique and potentially more hazardous than naturally occurring organisms and products of conventional chemical techniques. Since there are already established regulatory procedures and guidelines for the registration of chemicals and biologicals which are used as pest control products and as drugs, the issue is whether these can be appropriately used for the health hazard assessment and regulation of the products of biotechnology.

Regulation of the Product or Process? No evidence to date has suggested that a chemical which is manufactured by biotechnological processes would be inherently more toxic than the same product synthesized by conventional chemical methods. Therefore, it would appear reasonable to regulate the chemical regardless of whether it was synthesized by conventional chemical methods or through a biotechnical process, although from an occupational standpoint the manufacturing process and the assurance of its containment are also a concern. This paper, however, deals exclusively with the end product to which the public and the environment will be exposed. Any new chemical, regardless of the manufacturing process, is considered initially to be potentially toxic both to humans and to the environment and must be thoroughly tested to assess its safety before commercialization.

Existing Guidelines for Health Hazard Assessment.
(i) Pesticides. Pesticides have been regulated for many years and the primary function of the testing requirements is to ensure that the product does not pose an unacceptable hazard to human health or

the environment. No pest control product may be sold in Canada without prior registration under the Pest Control Products (PCP) Act which is administered by the Department of Agriculture. Under Section 9(2) a(ii) of the Regulations, the registrant is responsible for providing results of scientific investigations respecting the safety of the control product to persons occupationally exposed to it when it is manufactured, stored, displayed, distributed or used. Section 9(2) b(i) also requires, if the control product is to be used on food, that the Minister be provided with the results of scientific investigations respecting the effects of the control product or its residues when administered to test animals for the purposes of assessing any risk to humans or animals.

Data must be provided on the identity of the chemical active ingredient as well as of the contaminants (12-13) (Table I). These data requirements are sufficiently inclusive to be applicable to chemicals manufactured by biotechnological processes. It should be emphasized that different tests may be necessary to provide the data for genetically engineered products and that new tests may have to be developed to do so. Toxicity testing requirements to assess potential hazard to human health are outlined in T-1-245 (14) and are summarized in Table II. It should be noted that the technical

Table I. Registration Data Requirements (Chemicals)

Specifications	Residue Data
Analytical Methods	Environmental Chemistry and Fate
Manufacturing Methods	Environmental and Non-Target Studies
Quality Control Methods	Efficacy
Impurities	Sample of Product
Toxicology	

material, which would include both the active ingredient and contaminants, must be used in the tests. This would be equally important when testing biotechnology products where the spectrum of contaminants (e.g., cellular debris, including protein, membranes and nucleic acids) could be different, and different analytical techniques may be necessary. In addition, formulated products must also be tested to identify the potential impact of the other ingredients in the formulation. There are also requirements for testing for environmental hazard and for efficacy (Table II).

It therefore appears that the existing data requirements for assessment of hazard to human health from chemical pesticides manufactured by conventional chemical methods would be suitable for chemicals manufactured by biotechnological methods with the caveat that new tests may be necessary to fulfill the requirements.

(ii) Drugs. Regulations under the Food and Drugs Act are applicable to products of biotechnological processes, including rDNA, use of hybridomas for monoclonal antibodies, and continuous cell lines (15). These products are considered drugs with novel manufacture that must comply with Canadian (MRC) guidelines for the handling of recombinant DNA molecules and animal viruses and cells. Consider-

Table II. Toxicity Testing Requirements (Chemical Pesticides)

Acute Tests

- LD_{50} (oral, dermal, inhalation)
- Irritation (dermal, eye)
- Sensitization
- Delayed neurotoxicity

Short Term

- 90 day (oral, dermal, inhalation)
- 1 year feeding
- Delayed neurotoxicity (if acute test positive)

Long Term

- 2 year feeding
- Oncogenicity (2 species)

Special Studies

- Pharmacokinetic (absorption, distribution, excretion, metabolism)
- In vitro mutagenicity (point mutation, chromosomal aberrations, DNA repair)
- Teratogenicity
- Multi-generation reproduction
- Exposure

able attention to purification and to removal of extraneous substances and infectious agents is also required. Drugs produced by biotechnology may not be sold in Canada until licensed by the Bureau of Biologics.

(iii) Other Chemicals. Chemicals which may ultimately find their way into the environment are subject to regulation under the EC Act. The guidelines for data requirements for these chemicals are much less definitive than for chemicals which are subject to premarket evaluation and the onus is on the government to show that the chemical may pose a threat to health or the environment before the manufacturer can be required to submit more data. If a chemical manufactured by biotechnological processes has significantly different contaminants than the same chemical manufactured by conventional methods, there may be a need to require toxicity testing. This is likely to pose problems under the existing legislation.

Health Hazard Assessment for Organisms

The regulation of intentional release of living organisms as opposed to inanimate chemicals represents a different spectrum of potential hazards for man and the environment. Organisms, by their ability to

reproduce, can colonize a new habitat and displace a favourable species, leading to disruption of the balance of an ecosystem or to infection of a host. Although the major concern associated with chemicals is toxicity, areas of concern for organisms also include infectivity, virulence and hypersensitivity.

Are Organisms and Their Natural or Engineered Derivatives Comparable? The question is whether genetically engineered organisms which are being considered for intentional release should be considered as being different (more hazardous) than unmodified or naturally-occurring mutants.

The original approach to rDNA regulation, based on the premise that rDNA presented a unique hazard, led to adoption of guidelines for rDNA research in the U.S. (16), Canada (4), and elsewhere. There have been significant revisions of these guidelines, as experience has been gained and attitudes have evolved (17). At the present time, for example, because of knowledge of the safety of systems using *E. coli*, 90 per cent of rDNA research is exempt from guidelines.

In keeping with this revised approach, it has been argued from a biological perspective that organisms and their derivatives, whether natural or genetically engineered, are the same, and to regulate them differently would lead to discriminatory and inconsistent decision-making (18). However, a current proposal for regulation of biotechnology in the U.S.A. still attempts to categorize organisms on the basis of their origin or method of manufacture, discriminating against methods involving gene technology (19). Another approach would be to categorize organisms without resorting to the processes by which they arose, that is, a biological classification as opposed to technological distinctions.

Two biological categories can be described:

1. Organisms that have changes in their own genetic material (regardless of how the changes arose);
2. Organisms that contain foreign DNA (regardless of how the DNA was introduced).

The purpose of this distinction is to provide a system that does not rely on methodology. This classification is more consistent with principles of genetics and with the realization that rDNA techniques are really straightforward and relatively simple methods that do not have to be considered significant human intervention in a biological sense (18). Organisms in either category should be subjected to close scrutiny for potential health and environmental impact before approval for release.

What is known about natural processes in the cell (e.g., gene amplification, transposition, rearrangement, deletion, recombination and duplication) leads to the conclusion that any possible change in an organism's own genetic material could and probably has happened in nature. In fact, one could argue that it is preferable to rely on biotechnology for precise, limited and defined changes, in contrast to natural processes and conventional methods which can lead to unpredictable genotypic changes (20). Thus, organisms that have changes in only their own genetic material (whether arising spontaneously with either natural or artificial selection, arising through biotechnological techniques, induced by undirected mutagenesis, or with cell microinjection or microencapsulation of non-foreign DNA) logically can be placed within category 1.

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Organisms in category 2 should be considered separately because of the presence of foreign DNA, even though evidence does not yet exist to support the argument that foreign DNA may result in unique hazards. In fact, experience to date with the transfer of foreign genes has shown that a major obstacle often is expression of these genes in their new hosts, especially eukaryotes (21). A legitimate concern, however, is the accidental transfer of a closely-linked gene that might confer an unexpected trait on the new host. To allay this concern, the target DNA should be trimmed as closely as possible to conform only to the functional agent of interest. Absence of gene activity should be demonstrated when too much DNA is digested from either end of the target sequence and such evidence should be part of the data requirements for the characterization of the organism. How closely related are the donor and recipient organisms would also be a factor to consider for organisms in category 2. Another concern that often is raised is the potential for a harmless microorganism that contains a new gene accidentally to become pathogenic. On the one hand, pathogenicity depends upon the interaction of many genes (22) and, on the other, it seems unlikely that the gene for a desirable catabolic enzyme, for example, could code for a toxin or some other unwelcome product, just because it is in a new host.

In keeping with the above, it is proposed that evaluation of potential health hazards of organisms in both categories be on a biological (not a technological) basis. This approach is equally applicable to plant and animal breeding and interspecific crosses as well as to microorganisms. However, until more experience is gained in the rapidly growing study of heterologous systems (category 2), it seems prudent to consider that such organisms may be conceptually, if not biologically, different from organisms in category 1.

Examples of some of the data that probably will be required for the identification of any organism to be released include characterization of the species in general, including biochemical and pathogenic traits and relationships to indigenous organisms. If a new strain is involved, its history and derivation would have to be provided in detail, including the techniques used for its isolation. The new characteristics(s) of the strain would also be required, as well as the impact of the change(s) on target and non-target organisms. If the organism contains plasmids, the origins (if known) would be given, and they would be characterized for type, genetic content, stability and transmissibility to other cells and species. If genetic engineering was used to produce an organism, suggested additional information (23) would include the techniques used, full characterization of the modified genetic material and of control regions for gene expression, description of new traits including any loss(es) of normal function(s), and genetic stability. In addition, restriction maps delineating the functional ends of the gene and DNA sequencing data that show regulatory sequences and the open reading frame would be helpful in demonstrating how precisely the constructed segment corresponds to the desired function.

Regulation of Biological Control Products. Biological control products (organisms) also are regulated under the PCP Act. There

are currently 2 bacteria and 2 viruses registered in Canada. Although manufacturers have been required to submit data in support of their products for many years, it is only recently that these requirements have been formally written down (Table III). They will be issued through the Department of Agriculture as an R Memorandum in the near future (24).

Table III. Registration Data Requirements (Biologicals)

Specifications (strain, identification, amount of active ingredient)
Manufacturing Methods
Quality Control Methods
Impurities or Extraneous Materials
Certification of Ingredient Limit
Toxicology
Residue Data
Environmental and Non-target Studies
Environmental Fate
Efficacy
Sample of Product

In 1980, the United States Environmental Protection Agency (EPA) issued draft guidelines for registering pesticides which included data requirements for registration of biorational pesticides (25). Similarly, in November 1980, the World Health Organization (WHO) held an informal consultation to establish mammalian safety data guidelines for the development of microbial agents (26). Although numerous updated versions of the original 1980 EPA guidelines have been issued (27), the document remains in draft form.

In essence, guidelines developed by EPA, WHO and Canada, have focussed on a common set of issues. The guidelines initially proposed by EPA were to cover only those organisms which occurred naturally and were not known to be associated with mammalian infectivity or pathogenicity. They were based on the assumptions that:

1. hazards presented by microbial pesticides are inherently different from those of chemicals, and test methods and requirements should reflect this difference;
2. tests required to evaluate potential hazards to humans should be arranged in a hierarchical tier type of system. Negative data obtained at any level would obviate the need for further testing while positive results would implicate the next higher level of testing until the hazard of the organism is evaluated and quantified;
3. tier testing protocols should be developed to allow maximum opportunity for expression of any adverse effects; and
4. in general, any microbial control agents would have very restricted host ranges.

While the major area of concern for potential hazard associated with chemical use would be toxicity, areas of concern in

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the safety of microbial pesticides would include infectivity, virulence/toxicity and hypersensitivity. Infectivity studies would include investigations on persistence, replication, colonization and other host-parasite interactions. Typically, such studies involve examination of body weights, coat condition and colour, body temperature, hematology and histopathology of administration site and target organs. Virulence/toxicity studies would include assessment of direct injury at the cellular, tissue, organ or individual level, as well as potential long term effects. Immunological studies are carried out to determine if the test agent is capable of inducing either acute or delayed hypersensitivity of serious consequence. Current registration guidelines are based on evaluation of these endpoints. Tier testing for bacteria is therefore initially confined to acute studies by the oral, dermal, inhalation and intraperitoneal routes as well as testing for dermal and ocular irritation and dermal hypersensitivity (Table IV).

These studies include endpoints and investigations necessary to address the issues of persistence, infectivity, toxicity and irritation/sensitivity. Negative findings in all compartments at this stage of testing would obviate the need for further study while a positive finding would trigger further investigation, including multiple dose studies and quantification of persistence.

Similarly, initial tier testing for viruses would include acute testing by the oral, inhalation, intraperitoneal and dermal routes as well as dermal and ocular irritation studies and dermal hypersensitivity studies. Particularly important in virus testing, in sharp contrast to bacteria, is the need to establish the possibility of incorporation of the viral DNA into the host genome. These data requirements for naturally occurring biologicals are construed as being sufficiently encompassing to enable satisfactory evaluation of genetically engineered organisms, with the understanding that new tests may be necessary to obtain the data. One aspect that should be emphasized is the essentiality that detailed data be provided on the characterization of the new organism and this is an aspect that will require careful development.

Environmental Concerns

One major area of concern has been that of the uncertainty of the potential problems associated with the intentional release in the environment of genetically engineered organisms themselves. Is the manipulation of a gene so unique and fundamentally so different from natural mutational process to enable an organism to behave very differently in the environment? No evidence suggests that genetic engineering does have such a unique and profound influence on an organism; and no biological reason invokes suspicion that it would.

On the other hand, numerous examples have shown that introduction of organisms to new environments can have far reaching effects. Certain attempts at biological control of pests and accidental releases or importation are cited frequently as examples of the potential disasters that await any environmental releases of products of modern biotechnology. It is interesting to note when some of these events occurred: the mongoose was imported from Calcutta to Jamaica in 1872 to control rats in sugar cane fields; Kudzu vine was introduced to the southern U.S. to control erosion in

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Table IV. Toxicity Testing Requirements (Biological Pesticides)

Test	Species
Tier 1*	
Oral LD_{50}	Rat**
Dermal LD_{50}	Rabbit or Rat**
Inhalation LC_{50}	Guinea Pig or Rat**
Intraperitoneal	Rat or Mouse**
Primary Eye and Skin Irritation	Rabbit or Guinea Pig
Cellular Immune Response	Mouse
Hypersensitivity	Guinea Pig or Hooded Lister Rat
Tier 2	
Quantification of Persistence (most appropriate route)	Rat and Rabbit
Short-Term [90 day] (most appropriate route)	Rat or Mouse
Tier 3	
Chronic Oral	Rat
Oncogenicity	Mouse
Teratogenicity	Two species including one non-rodent

* If infectivity is clearly demonstrated in Tier 1 testing, organisms would not be considered for further development.

** Endpoints examined should include persistence, infectivity and toxicity

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the 1930's; gypsy moth imported for research purposes in the late 1800's was accidentally released from the lab; Japanese beetles in 1911, Chestnut blight in 1900 and Dutch elm disease in the 1920's, were all imported accidentally on nursery stock or lumber; and the starling got its start in North America in 1891 when about 80 birds were released in New York's Central Park (28,29). The question is whether past experiences have provided sufficient lessons to preclude repetition of these disasters. But more importantly, as emphasized by Brill (19), the environmental problems that arose from the intentional and accidental releases just mentioned bear no relation to the release of genetically altered organisms. The earlier problems did not arise from genetic change but rather by introduction of competitive species into new environments. To keep our perspective, it is instructive to remember that most North American crops were also imported from other parts of the world (20).

What we have learned from these examples is that, before its introduction into a new environment, regardless of whether the species is natural, a mutant, or genetically engineered, proper studies must be performed to assess its impact on its new environment. Repeated statements are heard on the need to develop further the science of predictive ecology to enable this assessment. This science should focus on the problem for any new situation and not just test whether genetically engineered organisms will behave differently than naturally-occurring and non-indigenous organisms, although it was this question that has stimulated recent concern about the potential impact of organisms in new environments. It may be that unrealistic assurances for "absolute safety" are sought without the proper perspective from previous events. There is no doubt that realistic and definitive questions must be asked and one approach may be to set stringent regulations which could be relaxed as information is gathered, as was the situation with the RAC and MRC Guidelines for rDNA research. If the primary concerns are based on potential ecological takeover, it is essential that all countries attempt to meet the challenge. Stringent regulations in Canada to limit release of organisms are meaningless, for example, if other countries do not regulate their industries effectively.

It is important to note and comment on two different philosophies regarding the desired persistence in the environment of organisms to be released. One approach involves an organism that already is successful in a given environment, modifying it (by whatever means) to possess specialized properties. For example, for pollution control, the organism not only would possess the required catabolic capability but also the traits needed for success in the ecosystem (30). This type of organism is chosen for its fitness in the target ecosystem so that it will persist, reproduce and increase in proportion to other indigenous species. These are the very properties that are of concern due to the possibility that the new organisms may be so successful as to become unwelcome pests.

An alternate philosophy is to prepare organisms that exquisitely perform the desired function but that will have limited and known survival in the environment. Organisms that are selected or engineered to be efficient specialists for a given function may have reduced fitness anyway, compared to wild type, due to increased energy requirements to perform the specialized tasks. Usually

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genetically engineered microorganisms do not compete successfully with their natural counterparts (18). Businesses that produce and sell such organisms will welcome the opportunity to replace organisms that perform their function but do not become established residents (31). Such an approach may not only be good for business but also allay fears about potential harm to the environment.

Another approach involves organisms that are genetically engineered (or otherwise produced), but the cells are killed before delivery, providing "bio-packages" of the active agent. An example is the toxin from *Bacillus thuringiensis* (Bt) encased in *Pseudomonas* (32). Such a product might be subject to regulation as a chemical, as a biological, or as a combination of the two. However, ecological impact obviously becomes much less a concern.

As mentioned above, and in most articles that discuss the environmental impact of altered organisms, relevant ecological studies are difficult to perform. The science of predictive ecology is unable to provide answers that can be used by regulators with the confidence that the environment can be protected, and some doubt that design of definitive, relevant tests ever can be achieved (20).

Summary

In the numerous articles on potential environmental or health problems of biotechnology, concerns that have been expressed seem to apply also to chemicals or naturally occurring organisms. Since biotechnological processes have had widespread use for years, and in some cases centuries, it is clear that most recent concerns of the past decade are really not about biotechnology but about the misunderstood and publicly maligned aspects of biotechnology that are referred to as genetic engineering (18).

Calls for strict and unique regulation of products of genetic engineering (11, 33) ignore the known hazard of introducing any organism into a new environment. It has been noted that in the rush to discuss conjectural hazards of rDNA technology, society has totally ignored other types of proven hazard (34).

Specific Canadian federal legislation allows regulation of biotechnological products for use as drugs and pesticides, and the suitability of other federal and provincial legislation for other types of products and processes is currently being evaluated, and certain deficiencies already have been noted in the federal legislation.

It has been proposed that chemicals that are inanimate products of biotechnology should not be considered unique and that the toxicity tests required for a chemical (using chemical pesticides as the example presented earlier) are adequate to regulate chemical pest control agents that may be derived from genetically engineered organisms or from other biotechnological processes. Products should be subject to regulation, although knowledge of the manufacturing process is important in that it may provide clues as to what impurities may be present. However, the use of rDNA, for example, should not require a different set of regulations to be used in the evaluation of a product's acceptability. There may be a need to develop new tests to provide the required data, however. The regulation of drugs by the Health Protection Branch is guided presently by this philosophy.

It has also been proposed that organisms can be classified conceptually according to whether or not they contain foreign DNA,

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but it is possible that this distinction will prove to have no biological significance for regulatory purposes. Aside from different requirements for characterizing the origin and expression of the foreign gene(s), the battery of infectivity/ toxicity tests appears to be suitable for evaluating the potential human health hazard of exposure to any microorganism. The tier approach for testing microbial pesticides that was described earlier appears to be adequate for viruses and bacteria that arise from any technical process.

Regulatory requirements that have not been addressed in detail concern the potential ecological impact of organisms proposed for release in the environment. Any organism may have a detrimental impact when introduced into a new environment. Therefore, for the sake of defensible and consistent regulation (18), any organism, regardless of the process used to produce it, should be evaluated as carefully as is possible before deliberate release.

Regulators routinely make decisions based on the best available information. This deliberate and cautious approach should not be distracted by the hypothetical questions that were raised in the climate of uncertainty ten years ago. The regulatory process should not be swayed, either by uninformed public opinion or by industry, but must be governed by the best evidence and be supported by sound scientific principles to provide protection for human health and the environment. In the present situation, it appears feasible that genetically engineered organisms and their products could be subject to the same type of regulations as naturally occurring biological organisms and chemicals.

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