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News Release

Bio-organic and Natural Products Chemistry Molecular Biology Workshop[1]

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INTRODUCTION

It was proposed at the October 1984 Meeting of the Bio-organic and Natural Products Chemistry Study Section that a workshop should be organized on the application of molecular biology to the areas of bioorganic and natural products chemistry. Molecular biology has gained sufficient maturity, despite its comparatively recent origin, such that scientists in the chemical sciences are seeking to apply these techniques to solve important problems in bio-organic and natural products chemistry. Specific techniques include site-directed mutagenesis, DNA sequencing, and gene cloning, which are useful in the production of altered proteins, analysis of DNA sequence specificity, and study of antibiotic biosynthesis, respectively. It is anticipated that the application of techniques from molecular biology will have a dramatic impact on areas reviewed by the Bio-organic and Natural Products Chemistry Study Section.

The workshop was organized by Drs. Laurence Hurley, John Kozarich, and Michael Rogers and held at Gerogetown University, Washington, D.C., prior to the June 1986 study section meeting. Approximately 60 scientists, including speakers (see Table 1), study section members, NIH staff members, and interested scientists predominantly from the Washington–Baltimore area, attended the workshop. A summary of the talks and discussions prepared by Laurence Hurley and John Kozarich appears in the following pages. Conclusions are summarized at the end. We wish to thank Dr. Carl A. Kuether of the NIGMS and Dr. Moreshwar V. Nadkarni of the NCI for their encouragement and support.

SUMMARY OF WORKSHOP

Principles and Tools in Molecular Biology

The recent explosion of advances in the chemistry and biochemistry of recombinant DNA technology has given bio-organic chemists and enzymologists a unique opportunity to design experiments whose execution would have been impossible several years ago. Drs. Shortle, Bittner, and Zoller accepted the task of providing the participants a primer on the basic principles and techniques of recombinant DNA technology.

Table I. Invited Speakers

Dr. David Shortle	Department of Biological Chemistry Johns Hopkins University
Dr. Michael Bittner	Biotechnology Division—Amoco Research Center Naperville, Ill.
Dr. Mark Zoller	Cold Spring Harbor Laboratory Cold Spring Harbor, N.Y.
Dr. Heinz G. Floss	Department of Chemistry Ohio State University
Dr. Richard Hutchinson	College of Pharmacy University of Wisconsin
Dr. L. Nicholas Ornston	Department of Biology Yale University
Dr. John Gerlt	Department of Chemistry University of Maryland
Dr. R. John Collier	Department of Microbiology & Molecular Genetics Harvard Medical School
Dr. John Essigman	Department of Applied Biological Sciences Massachusetts Institute of Technology

Dr. Shortle began with an overview of techniques. Probably the most important breakthrough in the field was the discovery of a wide variety of restriction enzymes. These sequence-specific enzymes cleave double-stranded DNA with a frequency that is dependent upon the occurrence of the particular DNA sequence which the enzyme recognizes. Since restriction enzymes vary greatly in the length and order of the sequence at which they cleave, a myriad of possibilities exists for the generation of DNA fragments of different lengths and containing different portions of genetic material. It is clearly possible, then, to excise specifically virtually any gene intact on a piece of double-stranded DNA. Of course, the specific identification and production of the desired fragment from all others require powerful cloning and screening methods. Cloning vectors are addressed in more detail later. Once the restriction fragments have been cloned en masse and colonies containing the cloning vector have been detected, screening methods must be employed to determine which, if any, of the colonies contain the DNA fragment in question. Screening methods that have been most frequently employed are analyzed for functional activity (i.e., if the gene

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product is an enzyme), DNA sequence homology by hybridization to an oligonucleotide complementary to a sequence in the gene, and reactivity of the gene product to an antibody specific for it. Clearly, without a good screen the probability of identification of a clone containing the desired gene is extremely low.

Once the desired clone has been isolated, a number of chemical techniques exist for the characterization of the DNA fragment. Fraction techniques are based largely upon gel electrophoresis. Agarose gels are usually employed when a resolving power in the 200- to 50,000-base pair range is required, while polyacrylamide can resolve 1–2000 base pairs. When combined with ^{32}P detection methodology and the DNA sequencing chemistry developed by Maxam and Gilbert and by Sanger, picogram amounts of DNA can be thoroughly characterized.

Dr. Bittner reviewed the large bank of diverse cloning vectors which have been developed. Most of these vectors have been constructed so as to give the scientist an enormous number of options for the expression of his/her favorite gene. Cloning vectors, in general, contain a marker function, a region containing genetic information for host-specific maintenance, and a region responsible for replication. *Escherichia coli* vectors fall into two broad classes: those that insert into the chromosome (chromosomal maintenance) and those that provide plasmid maintenance of the gene. A host of resistance markers, promoters, ribosome binding sites, and signal peptides to tailor individually the expression and localization of the gene and gene product is available. A similar array of vectors has been developed for yeast and for mammalian systems. New vectors are being developed so rapidly that most bio-organic chemists will probably find a collaboration with a molecular biologist most convenient.

Dr. Zoller's presentation dealt with the chemical synthesis and uses of DNA. Three approaches are currently used for the sequential formation of phosphodiester linkages: Khorana's phosphodiester method, the phosphotriester chemistry developed by Letsinger and Oglivie, and the phosphoramidite approach of Letsinger and Caruthers. Solid phase supports in favor are silica gel and control pore glass. Automation of the chemistry has developed to the extent that at least six companies offer DNA synthesizers at a range of prices. Capabilities are such that these machines can generate 0.2–10 μmol (50 μg–1 mg) of pure product in a short period of time. The preparation of 60- to 80mers is becoming reasonably routine and 90- to 125mers have been reported. Machines generally employ the phosphoramidite chemistry and a number of other useful capabilities, such as the introduction of modified bases at specific sites, may be incorporated into the system. Procedures for the purification of the oligonucleotides depend upon the length of the material. High-pressure liquid chromatography appears to be the method of choice for less than 25mers. Reverse-phase and ion-exchange chromatography have both been used, and speed and high capacity are important advantages. For longer oligonucleotides, polyacrylamide gel electrophoresis is preferred. Impurities that are one base shorter than the desired oligomer are easily separated, and many different samples may be purified on a single gel. The low capacity of these gels is a disadvantage. It is clear that nearly any possible oligonucleotide is within the synthetic grasp of chemists.

Application of Molecular Biology to Natural Products Research

Several aspects of natural products research are amenable to techniques from molecular biology. Drs. Floss, Hutchinson, and Ornston developed this theme drawing from specific examples of current problems that have been solved using recombinant DNA technology and pinpointing areas for future development. Dr. Heinz Floss presented an overview of the opportunities for the natural products chemist through recombinant DNA technology.

Recombinant DNA technology provides the means to isolate and modify proteins that are useful for the production of new biosynthetic products. For example, isopenicillin N synthetase has been cloned by workers at Eli Lilly company, and in collaboration with the Baldwin group at Oxford a wide variety of new β-lactams has also been gleaned from this isolated enzyme system. Possible advances in this area include the modification of enzyme structure to provide proteins with improved properties for both *in vitro* and *in vivo* purposes. The construction of microorganisms that produce new biosynthetic products is an exciting possibility. With the increasing popularity of the use of enzymes by synthetic chemists, the production of modified proteins with improved catalytic and physical–chemical properties is also an attractive possibility.

The study of the mechanism of action of physiologically active compounds such as antibiotics and antitumor agents that react specifically with known receptors such as enzymes and nucleic acids can be greatly aided by the availability of these purified receptors in amounts that can be studied by physical methods such as nuclear magnetic resonance (NMR) and X-ray crystallography. The isolation of these receptors in sufficient amounts for these types of studies is now possible through recombinant DNA technology. For example, restriction enzyme fragments can be isolated to examine ligand binding and shorter oligomers can be synthesized for drug binding and characterization studies.

The identification of new physiologically active compounds and optimization of yield of secondary products are amenable to recombinant DNA technology. New assay systems that rely upon purified receptors or the construction of genetically altered screening organisms are examples of opportunities in this area. In the fermentation area the random screening of mutants of microorganisms for increased yields of valuable products will be replaced by more rational approaches that release products from tight biochemical control mechanisms. Important questions relating to the reasons why microorganisms and plants produce secondary products and how these biosynthetic pathways have evolved are amenable to recombinant DNA technology. Last but less obvious are opportunities to probe fascinating problems in coevolution in, for example, systems where insects require plant natural products.

Probably the area of natural products research which has benefited most from molecular biology is antibiotic production by *Streptomyces* species. Dr. Hutchinson, a natural

products chemist who has made a considerable investment in applying techniques from molecular biology to study *Streptomyces* antibiotic production, reviewed both the techniques and the application of this technology to this work. A major problem in gene cloning in *Streptomyces* is obtaining appropriate transformation procedures, and various approaches were discussed. Although the study of *Streptomyces* genetics is still relatively new, considerable progress has been made in obtaining suitable vectors and studying gene expression in *Streptomyces*. This is an exciting area for natural products chemists and, along with the more recent chemical approaches to studying biosynthesis, is likely to yield important information that will impact on both basic and applied research questions.

Dr. Nicholas Ornston concentrated on the use of recombinant technology to study the biodegradation and biotransformation of xenobiotic substances. This approach offers many possibilities such as providing the enzymes to study reaction mechanisms, enzyme structure, the production of biodegradative enzymes, and their organization, control, and evolution. Methods for the construction of recombinant plasmids and the use of transposons to probe these problems were reviewed. Last, the exciting possibility that gene conversion was responsible for evolutionary diversity was discussed.

Application of Molecular Biology to Bio-organic Chemistry

Site-Specific Mutagenesis

The techniques described above have made possible the incorporation of a single amino acid substitution at any position in a protein. Such a change may be made in order to test a mechanistic hypothesis concerning the function of the native amino acid during normal protein action or to alter specifically the properties of the protein whether they be kinetic parameters of enzyme catalysis or other factors such as thermal stability or alteration of subunit interactions. Drs. Gerlt and Collier addressed these applications by providing the workshop with results of experiments currently ongoing in their laboratories.

Dr. Gerlt has been using site-specific mutagenesis as a probe in studying the mechanism of action of staphyloccal nuclease, a small phosphodiesterase. A 1.5-Å X-ray crystal structure exists that provides fairly detailed information concerning which amino acid functionalities may be important to substrate binding and catalysis. One particular residue upon which Gerlt has focused is the active-site glutamate, which has been implicated as a general base during the hydrolysis of the phosphodiester. Specific mutation of this residue to an aspartate resulted in a 10^{-3} decrease in V_{max}/K_m. Since the aspartate carboxyl group is contracted away from the active site, this result may suggest that general base catalysis by glutamate is worth a factor of 10^3 in catalysis. This assumes, of course, that the mutant enzyme is structurally identical to the native enzyme. Since staphyloccal nuclease is small enough to be amenable to ^{1}H NMR analysis, Gerlt has used this technique as a sensitive probe to assess environmental changes of specific amino acid residues upon single amino acid substitutions. His findings suggest that the assumption of structural integrity may not be solidly founded. He finds that a variety of residues, some of which are well removed from the point of mutation, exhibits significant changes is the chemical shift of proton resonances. While this suggests that environmental, and therefore structural, changes do occur, it is difficult at the present time to quantitate their magnitude and significance. Overall, unless one has good structural backup to complement mutagenesis findings, there appears to be a real possibility that the results could be misinterpreted.

Dr. Collier's use of site-specific mutagenesis has been directed toward an understanding of the mode of action of diphtheria toxin. Diphtheria toxin catalyzes the ADP-ribosylation of the target protein, EF-2, which ultimately leads to the physiological effects of the disease. Enzymological studies have shown that a portion of the toxin binds NAD and is responsible for the ADP-ribosyl transfer. Photoaffinity labeling studies with NAD have revealed that a photochemically induced insertion of the nicotinamide moiety into a specific glutamate occurs, suggesting that this residue may form an ion pair with the nicotinamide cation during binding of NAD. In order to test this hypothesis, the specific glutamate-to-asparate mutant was constructed and was found to be inactive in the ADP-ribosylation reaction. This supports the idea of a close ionic interaction, assuming that the mutation did not lead to significant structural changes (vida supra). A potential medical application is the use of genetically engineered inactive toxin as a vaccine.

Site-Directed DNA Adducts

An old but important problem in studies on mutagenesis and carcinogenesis is relating the structure of the DNA lesion to the genetic change which it causes. Most agents produce a multiplicity of DNA modifications, only some of which may be biologically significant. Without methods to evaluate single types of lesions in assigned genome sites, obtaining definite information of this type has been elusive. Dr. John Essigmann reviewed the procedures available to produce chemically defined systems for studying DNA lesions which involved a combination of oligomer adduct synthesis and genetic engineering. This technology was first applied to simple base modifications such as methylation but is now being applied to more complex substrates such as cisPt. The resulting defined lesions on DNA can then be used for *in vitro* and *in vivo* experiments to study replication and repair.

CONCLUSIONS

It is clear that techniques from molecular biology will play an important role in research related to the bio-organic and natural products chemistry areas. In some cases, these techniques offer improved approaches to solving problems over previous procedures. For example, the use of genetic engineering permits the preparation of chemically defined systems for studying questions related to the genotoxicity of DNA modifications. In contrast, some techniques such as site-specific mutagenesis offer opportunities to investigate questions such as the function of native amino acids in mechanistic hypotheses not previously amenable to scientific inquiry.

For medicinal and natural products chemists wishing to

apply techniques from molecular biology to problems of current interest, it may be advisable either to set up a collaborative research program with an experienced investigator or to take a sabbatical in an appropriate laboratory unless the investigator has had prior experience with these techniques.

Finally, it cannot be stressed enough that investigators wishing to stay on the forefront of research in the bio-organic and natural product chemistry must be sensitive to the appropriate application of techniques from molecular biology to certain problems in this area. A historical equivalent to the avoidance of these new techniques is perhaps the refusal 20 years ago to become acquainted with NMR as a technique for structural elucidation.