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MARINE BIOTECHNOLOGY: BASIC RESEARCH RELEVANT TO BIOMATERIALS AND BIOSENSORS

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Committee on Biotechnology Applied to Naval Needs
Commission on Life Sciences
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PREFACE

The Committee on Biotechnology Applied to Naval Needs was formed in the Board on Basic Biology within the National Research Council's Commission on Life Sciences in response to a request from the U.S. Navy for advice on the applications of biotechnology to meet naval needs. Initial discussions of this project included both the Naval Air Systems Command (NAVAIR) and the Biological Sciences Division of the Office of Naval Research (ONR), which decided that the Research Council committee should focus on biomaterials, biosensors, and molecular electronic devices. In subsequent discussions with ONR, the project's sponsor, it was agreed that the committee's study should be focused on basic research for possible support by ONR rather than on development. The charge to the committee was narrowed to an examination of basic research that may lead to developments in biotechnology in only two fields: biomaterials and biosensors. The sponsor decided that the committee would not review ONR's ongoing program of research support.

The committee organized two conferences to explore the two major subjects of its study. The first of these, on biomaterials, was held in Cambridge, Massachusetts, on September 19-20, 1983; the second, on biosensors, was held in Washington, D.C., on May 7-8, 1984. A report on each of these was submitted to ONR after the conferences.

The committee sought advice from a variety of sources, including ONR, to develop a working definition of biosensors and biomaterials and to identify scientists studying diverse aspects of those subjects who could present papers describing the most important scientific problems in their disciplines and current approaches to their solutions. In addition, these scientists were asked to indicate directions that basic research should take over the next several years. Thus, the emphasis of the conferences was placed less on the nature of specific biomaterials or biosensors but rather on biological systems or analogs thereof and interactions between these systems.

For the purposes of this report, biomaterials are defined as substances produced by biological systems or synthesized from biologically produced components. The conference devoted to this

topic was divided into four sections: genetic engineering; proteins and polysaccharides; membranes, including interactions between biological and nonbiological materials; and future directions for basic biological research in these areas.

Biosensors are defined as substances or devices that, through biological functions, generate signals in response to a specific material or condition. The conference on this topic consisted of sessions covering receptors, enzymology and immunology, DNA as a detector, and the development of synthetic analogs to natural biological reactions.

In both conferences, the participants provided brief written synopses of their talks. These, together with their oral presentations, were used by the committee to develop the conference summaries included in the first two reports. These summaries also served as the foundation for the committee's discussion of the important areas of biology meriting support from ONR. In the present report, the committee has synthesized the findings of the first two reports, added material from further literature reviews, and recommended areas in basic research relevant to biomaterials and biosensors to be supported by ONR.

The committee members who prepared this report and summarized the conferences are listed on page iii. The conference participants are listed at the end of the report. We wish to thank David Policansky and Agnes Gaskin of the Commission on Life Sciences staff for their able assistance and acknowledge the ONR for its support. The committee is grateful to the participants in the conferences for their presentations and helpful discussions and for providing us with literature for use in our study.

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Chapter 1

EXECUTIVE SUMMARY

In response to a request from the Office of Naval Research (ONR), the Committee on Biotechnology Applied to Naval Needs (CBANN) of the National Research Council (NRC) held two conferences and recommended areas of basic research for funding by ONR. Proceedings of the two conferences--the first on biomaterials and the second on biosensors--were presented in separate reports submitted to ONR in 1984 and 1985. The subject matter of both conferences is reviewed, summarized, and discussed in the present report, which also contains general and specific recommendations for research to be funded by ONR.

The development of marine biotechnology is dependent on advances in basic research. Thus both conferences focused on basic science that could be expected to lead to developments of biomaterials and biosensors and their applications. After reviewing all relevant information, the committee concluded that there are two broad areas that need to be elucidated: in biomaterials research, it is the relationship between structure and function of biological molecules; in biosensor research, it is the transduction of biochemical reactions to an electrical signal in real time. It therefore recommends that the ONR support basic research in these areas, [redacted]

The following paragraphs summarize the committee's major findings under three headings: general categories, including mechanisms of funding and types of groups to be funded; specific areas of research; and techniques, including instrumentation. Major recommendations in these areas are given below.

GENERAL

Increased ONR support of basic research in the biological sciences would lead to the establishment of a group of investigators who would serve as a resource for advice on specific topics of interest to ONR. Indeed, ONR is in a unique and enviable position to catalyze important advances in the basic sciences and to build a foundation of knowledge [redacted] the United States.

The committee recommends that ONR continue to support basic research in marine biology, especially for single investigators and research teams. Development and exploitation of the results from such research are indeed also important, but they are proceeding successfully elsewhere. For that reason, and because they were not included in the ONR charge to the committee, recommendations for development activities are not offered in this report.

Multidisciplinary projects have great potential value to marine biotechnology because they bring together individuals with different perspectives on research problems. The often fruitful interactions of physicists, chemists, marine biologists, biochemists, microbiologists, and others could lead to breakthroughs in basic research in areas related to biosensors and biomaterials. For this reason, the committee recommends that ONR consider proposals submitted by groups of investigators from diverse disciplines, perhaps under the leadership of one principal investigator. Cooperative undertakings among diverse research groups in universities and in industry should be encouraged.

SPECIFIC RESEARCH AREAS

Five areas of basic research have exciting and useful potential for the acquisition of knowledge concerning biomaterials and biosensors: nucleic acids, proteins, polysaccharides, synthetic analogs, and holistic functions of marine organisms. Because major scientific discoveries cannot usually be predicted, there was some reluctance to pinpoint specific research projects from the long list identified by the committee. Nonetheless, the following areas stood out as being highly promising and worthy of ONR support:

- For nucleic acids: structural chemistry and conformation, molecular genetics, and techniques of genetic engineering.
- For proteins: structure and function relationships, immunological detection, and exploration of unusual enzymes.
- For polysaccharides: structure and function relationships, understanding and cataloging their specificities, and a search for new and unique polysaccharides.
- For synthetic analogs: specificity of their biological action and activity in unusual microenvironments, including nonaqueous solvents.
- For whole organisms: unusual organisms, especially those from extreme environments, sensory detection and communication, and systematics and ecology of [redacted]

TECHNIQUES

Research is often advanced by the development of new techniques and instruments. The committee believes that advances in the following techniques are vital for the basic research recommended above and ultimately for [redacted]gy: transducers to convert the antigen-antibody reaction to an electrical signal in real time; use of DNA probes; amplification of signals; methods for culturing and maintaining unusual organisms, especially those from extreme environments; improvement of instrumentation and refinement of specific techniques for determining molecular structure; and specific methods for cleaving polysaccharides.

In summary, biomaterials and biosensors have important potential applications in marine biotechnology. Thus, basic research with a focus on the [redacted] providing the necessary foundation for future developments in these rapidly changing areas should be useful to the Navy and therefore funded by ONR.

Chapter 2

INTRODUCTION

Biotechnology is not new, since the techniques of microbiology and genetics have been applied in food technology and agriculture for centuries. However, promising new technologies such as recombinant DNA and hybridomas have recently attracted special attention. Because of its potential, biotechnology is of great interest to universities, national governments, and a variety of industries. Recent publications, including those in the lay press, business and government reports, and scientific literature, indicate the depth and breadth of this interest (Abelson, 1983; Combs, 1984; Congressional Research Service, 1984, Crafts-Lighty, 1983; Klausner, 1985; National Research Council, 1982, 1984a,b; Office of Technology Assessment, 1984; Tucker, 1985; U. S. Department of Commerce, 1984; Zaborsky and Young, 1984).

Biotechnology, especially marine biotechnology, is a vital concern of the Office of Naval Research (ONR). In addition, ONR has a tradition of funding basic research leading to developments critical to naval needs. Because biotechnology encompasses many fields of science and engineering, however, it is a difficult subject to survey comprehensively. Moreover, some promising aspects of biotechnology will be realized only far in the future. For these reasons ONR has sought the advice of the National Research Council to identify areas of basic biological research that may lead to developments in the biotechnology of sensors and materials appropriate to the needs and interests of the U.S. Navy.

The committee's charge defined the general focus of its study but did not specify that the resultant recommendations be based on ONR's current research program. Instead, the committee was asked to look at aspects of basic research with potential applicability to the development of biosensors and biomaterials. In this third and final report, the committee has identified five major topics relevant to biosensors and biomaterials that merit research support by ONR. Some of the recommendations were included in the first two reports; others are new, arising from additional review of the literature and further discussions of the committee.

The committee believes that the development of marine biotechnology is dependent on advances in basic research. Because the Navy's mission is so closely linked to the world's oceans, it has a unique opportunity to take a leading role in supporting the basic research that will lead to developments in areas such as biomaterials and biosensors. This role should not be confined to preservation but should include exploitation as well. Support is especially needed for research on marine organisms, not only because they are an understudied genetic pool, but also because their adaptations to the marine environment are potentially applicable to marine biotechnology.

Because of the vastness of the subject matter, the committee did not exhaustively investigate all areas of potential interest to ONR, e.g., whole organism sensory physiology. It also did not wish to limit ONR's flexibility in choosing potentially productive projects by establishing rigid research priorities. Certain areas have been highlighted, however, to provide ONR with guidance in its selection of basic research that might lead to advances in biotechnology.

CHAPTER 3

NUCLEIC ACIDS

In its very structure, which involves two complementary strands in cells, deoxyribonucleic acid (DNA) provides an explanation for heredity and is the repository of all genetic information carried by the chromosomes (Watson, 1976). However, not all nucleic acid is informational in character nor is all the informational nucleic acid found in the chromosome. The study of nucleic acids, i.e., DNA and RNA, is of central importance to the understanding and the practical application of biological systems such as for biosensors or biomaterials.

STRUCTURE OF NUCLEIC ACIDS

Nucleic acids are linear polymers composed of a relatively small number of building blocks or monomers called nucleotides. There are only a few types of nucleotides, and the linkages between them are all of the same type. DNA and RNA are chemically different. Both contain four different nucleotides differing with regard to the sugar, which is ribose in RNA and 2-deoxyribose in DNA. Each nucleotide contains three covalently linked components: a nitrogenous base (either a purine or a pyrimidine), a 5-carbon sugar (ribose or deoxyribose), and a phosphoryl group. In DNA there are four commonly occurring deoxyribonucleotides (adenine, guanine, cytosine, and thymine). In RNA there are four commonly occurring ribonucleotides, three of which have the same nitrogenous bases as those in DNA. The fourth, uracil, replaces thymine in RNA.

REPLICATION, TRANSCRIPTION, AND TRANSLATION

The two DNA strands each serve as a template for making a new partner strand, and this is crucial to the role of DNA as hereditary material. The genetic information is contained in the nucleotide sequence of each strand, and since the sequence is exactly complementary to that of its partner strand, both strands actually carry the same genetic information for the replication process. There are only four different nucleotides, but the amount of information and

the biological variety that they can impart are great: a typical animal cell contains a linear meter of DNA (3×10^9 nucleotides). Although the principle underlying DNA replication is simple, the machinery is complicated and involves many different proteins and enzymes.

In the first step in protein synthesis, specific regions of DNA (i.e., genes or coding regions) are copied into RNA. RNA thus retains all the information of the DNA sequence from which it was copied as well as the base-pairing properties. RNA is single stranded, however, and messenger RNA (mRNA) is physically moved and read from one end to another during protein synthesis on the ribosome--a complex of almost 100 different proteins associated with several structural rRNA molecules.

The mRNA nucleotide sequence is read in serial order in groups of three. Each triplet of nucleotides, called a codon, specifies one amino acid. Since RNA is a linear polymer of four different nucleotides, there are 4^3, or 64, possible codon triplets. Since there are only 20 amino acids most amino acids may be specified by more than one codon.

Codons of mRNA do not directly recognize the amino acids that they specify as an enzyme recognizes its substrate. Instead, small RNA molecules known as transfer RNAs (tRNAs), each of which is 70 to 90 nucleotides in length, serve to recognize both an amino acid and the corresponding triplet.

RECOMBINANT DNA TECHNOLOGY

Our increased understanding of molecular biology has led to new views of genetics and all biology and heralds the maturity of recombinant DNA technology. Because of recent developments in this field, there are now powerful and novel approaches to understanding the complex mechanisms by which eucaryotic gene expression is regulated and to the adoption of new methods for determining protein amino acid sequences. The large scale and economical commercial production of proteins--hormones, vaccines, enzymes--has almost been achieved.

Recombinant DNA technology relies on (1) the specific cleavage of DNA by restriction nucleases, permitting the isolation of desired sequences or genes; (2) nucleic acid hybridization, making it possible to identify specific DNA or RNA sequences; (3) DNA cloning, in which a specific DNA sequence is incorporated into a rapidly replicating genetic element (plasmid or virus) so that it can be amplified in bacteria, yeast, or other eucaryotes; and (4) DNA sequencing techniques as developed by Sanger et al. (1977) and by Maxam and Gilbert (1977).

CONFORMATIONAL VARIANTS OF DNA: DOUBLE HELIX STRUCTURE

There may be a considerable variation in the conformations adopted by double-helical polynucleotides due to the inherent flexibility in the pyranose ring and the degrees of freedom generated by six rotatable single bonds per residue (Record et al., 1981; Zubay, 1983). Thus B-DNA, which has 10 base pairs per turn and planes nearly perpendicular to the helix axis, is believed to be the major structure of DNA in solution. The A form, which contains less water, has approximately 11 bases per turn and planes tilted about 20 degrees to the helix axis.

The structure of the recently observed Z-DNA is strikingly different from other DNA double helices with base pairing. It has a left-handed rather than right-handed twist and is considerably slimmer, containing 12 base pairs per turn (Wang et al., 1979). Recent observations suggest that left-handed helical DNA may exist in specific regions of naturally occurring DNA and that it may play a role in regulating gene expression.

SUPERCOILED DNA: TOPOLOGY OF DNA STRANDS AND POLYMERASE ACTION

In DNAs that are topologically constrained by being circular or complexed to proteins so that the ends of the DNA cannot rotate freely, either right-handed (negative) or left-handed (positive) supercoiling or twisting of the DNA loop may occur. The more extensive the supercoiling, the more compact the molecule, making it possible to separate molecules with different degrees of coiling and to study factors responsible for supercoiling and its relaxation (Revet et al., 1971). This has led to the discovery of several kinds of enzymes called topoisomerases, which can either reduce or increase the tightness of coiling (winding number) of a supercoiled helix (Gellert, 1981). The enzymes can break and rejoin DNA repeatedly without any added energy supply or cofactors, thereby apparently conserving the energy of the DNA phosphoester bond.

Topoisomerases are also believed to be responsible for preventing the tangling of DNA during transcription of both DNA and RNA. Since polymerases act on only one (the leading) of the two DNA strands, the duplex must open, and for every 10 base pairs replicated at this fork, the parental double helix must make one complete turn about its axis. By forming a swivel in the helix, DNA topoisomerases circumvent this problem.

IMPORTANCE OF NUCLEIC ACIDS FOR BIOTECHNOLOGY

Studies of nucleic acids have relevance and applications in many different areas. The key direct role of nucleic acids in heredity and in the synthesis of macromolecules, especially proteins, means that

they are pivotal both chemically and biologically in terms of the basic aspects of cell metabolism, growth, and replication.

The recent rapid developments in genetic engineering have emerged from the successful applications of knowledge concerning fundamental aspects of nucleic acid structure and function. In particular, the discoveries of restriction endonucleases and polynucleotide ligases (Nathans and Smith, 1975) were key, but unpredicted, fundamental prerequisites for all genetic engineering. Basic knowledge and techniques from studies of viral and bacterial genetics were and continue to be equally important.

Biosensors

Related to their informational role but quite distinct is the specificity of nucleic acids for recognition at the molecular level. The binding and the character of the recognition are qualitatively different from substrate and enzyme or antigen and antibody: in nucleic acids the complex involves a polymeric molecule, one in which each of the individual monomers in the strand contributes to and is essential for the binding. Thus DNA probes, through which a specific nucleotide sequence (i.e., gene) can be detected, have considerable potential for application in biosensor development (Moseley et al., 1980; Old and Primrose, 1981).

The study of DNA protein-specific interactions and their role in the regulation of gene expression is a fast moving area of research (Takeda et al., 1983; von Hippel et al., 1983). These specific macromolecular interactions, like the ones between immunoglobulins and their substrates, may have applications in the development of sensors.

Biomaterials

The importance of nucleic acids as structural molecules should not be overlooked. These biopolymers are unique in many ways. Because of the enormous length of single molecules and their existence in the uncoiled or partially uncoiled state under certain conditions, the physical properties of nucleic acid solutions are unusual. Covalent bonds may be broken mechanically by sheer forces (the syringe effect). Their electronic states and luminescence are other properties with potential for practical application (Callis, 1983). The classic double helix continues to provide an important general model for structural systems. Supercoiling and the unusual enzymes associated with the phenomenon introduce topological questions related to molecular conformations. The committee expects that unusual and potentially important biomaterials can be developed from knowledge of these molecules or from the principles derived from knowledge of their structures.

Chapter 4

PROTEINS

Ever since the elucidation of the double helix by Watson and Crick (1953), much attention in biology has been focused on the DNA molecule. In modern computer terminology, the DNA molecule can be regarded as the software of a cell and the protein as the hardware. All the information necessary to make proteins is stored in the DNA molecule, and the machinery necessary to carry out the process is vested in the very same proteins.

Proteins are both chemically and functionally much more complicated than DNA. DNA is made from four monomers that are chemically very similar, whereas proteins consist of 20 monomers--the amino acids--each with distinct chemical properties. Lehninger (1975, p. 6) has estimated that there are approximately 10^{12} different protein molecules in nature. One could make that many entities by using only 10 amino acids, but normally there are a few hundred amino acids in a peptide chain. Thus, the number of possible proteins--20^{200}--is much greater than the number of atoms in the universe.

Proteins have an enormous range of functions in nature (Lehninger, 1975, p. 64). To understand the function of a protein molecule, it is necessary to know the three-dimensional structure of the peptide chains. Customarily, four levels of protein structure organization are distinguished. The primary structure is the amino acid sequence in a peptide chain. The secondary structure encompasses some common conformational patterns recurring in many proteins, such as the alpha helix and beta sheet. The tertiary structure is the complete three-dimensional structure of a peptide chain. The quarternary structure refers to the spatial organization between two or more peptide subunits in a protein.

It is generally accepted that the conformational folding of a polypeptide chain is determined by its primary structure. The chain may fold into a state with minimum free energy, or it may not, since the peptide chain folds from one end as it is made. In principle, then, if the primary sequence is known it should be possible to predict the three-dimensional structure of a protein with an approach relying only on the speed of the computer, rather than on insightful

analysis. The following calculations demonstrate the magnitude of the brute force approach needed to solve such a problem.

Although the peptide bonds are planar, two of them can rotate around each other through the alpha-carbons. If only four positions are available between two adjacent peptide bonds, there would be 4^{200} or roughly 10^{40} configurations for a peptide chain 200 amino acids long. If a computer could evaluate the energy for one position in 10^{-6} second, it would evaluate 2×10^{13} configurations in a year, and the time needed to evaluate all the configurations would exceed the age of the universe. If special purpose analog computers could be developed, or if parallel processing digital computers become available, it is conceivable that the time limitation could be overcome.

Proteins constitute roughly 50% of the dry weight of a cell and are intimately involved in all the cell's functions. Thus, to understand any function on a molecular level, it is necessary to understand the interactions between proteins as well as those between proteins and other molecules. The following general statements apply to all proteins.

The ultimate goal in protein chemistry is to equate the structure of a protein with its function. At present, unfortunately, the only way to obtain atomic resolution of the structure of a protein is by x-ray diffraction, which requires that the protein be crystalline. The structures of perhaps 200 proteins have been determined in this way (Alberts et al., 1983, p. 113). Because of the great chemical variability in the ability of proteins to form crystals, the characterization of their structure is more art than science. It requires patience, skill, perseverence, and money. Even with pure crystals, the technique is difficult because of the "phase" problem, i.e., x-ray diffraction measures only the intensities of the diffracted rays, but not their phases, which are needed for determining structure. If x-ray lasers are ever developed, it should be possible to measure phases with them.

To a limited extent, protein molecules can be visualized in the electron microscope. Although the microscope has close to atomic resolution, however, the contrast of the protein molecules is not sufficient. A new approach in which image enhancement is used to look at electron diffraction from two-dimensional crystals offers some promise (Uzgiris and Kornberg, 1983). Attempts to adapt unconventional microscopes, like the field ion microscope, for this purpose have had limited success (Muller and Tsong, 1969, pp. 289-292; Panitz, 1983; Panitz and Ghiglia, 1982).

An elusive goal is to predict the structure of a protein when the primary structure is known. Since the brute force approach is clearly impossible, much thought and experimentation have gone into trying to understand some general principles of polypeptide folding. Although progress is being made, no general approach has been developed

(Richardson, 1981). A second complication is the modern view of protein molecules as dynamic structures with several "breathing motions"--structures that spend time in a variety of similar configurations (Karplus and McCammon, 1981).

Through genetic engineering, it is now possible to create a totally new long chain polypeptide or, on a more modest scale, to make systematic amino acid substitutions in existing proteins. However, because of the enormous difficulties in predicting peptide conformations, no general a priori design principle exists, and such processes rely on trial and error. Nevertheless, this is a very active research area and a necessary process to gain more understanding of protein folding.

BIOSENSORS AND BIOMATERIALS

To understand many biological functions it is necessary to understand the interaction between molecules, especially proteins. Such knowledge is also necessary in the development of biosensors or biomaterials or in deciding whether or not such a project is feasible. The following discussion of the biochip provides an example of basic research with a potential for practical application but points out the difficulties encountered in putting ideas into practice.

Biochips

A unique feature of living organisms is the self-organization of many complicated structures, such as large enzymes or viruses. This ability cannot be matched by ordinary manufacturing technologies, primarily because protein molecules and other building blocks of life are small enough to be transported by diffusion and are present biologically in small compartments. Such small structures have not commonly been used by industry to manufacture commercial products, although the electronics industry is rapidly approaching this small scale. As a result, there has been speculation about the use of biological macromolecules as biochips--i.e., electrical circuit elements and self-assembling computers. It is important to remember, however, that biological molecules are insulators and conduct electricity very poorly, that they are very unstable compared to silicon, that proteins cannot be designed today, that life processes are slow because they depend upon diffusion rather than on the speed of light, and that silicon is probably cheaper than biological macromolecules. [redacted]

IMMUNOLOGY

A research area offering great promise in the development of biosensors is immunology. Antibodies are proteins made by a class of

lymphocytes, the so-called B-cells, in response to an invasion of foreign matter into the body. Because of the prime importance of the immune system in fighting disease, the system has been and is under intense study, and the humoral (B-cell) part is at least partially understood (Weissman et al., 1978, pp. 41-64). In humans there are five classes of immunoglobulins of which IgG and IgM are the most common. Since antibodies are very specific in their interactions with antigenic determinants, and since they can be made to interact with virtually any molecule, this system has great promise as a general biosensor. In the medical community, immunology tests are widely accepted. For example, all donated blood in United States is screened routinely for hepatitis, and screening for syphilis is generally required before marriage.

Antibodies used in these tests are normally obtained by injecting rabbits or goats with the antigen. Even though the serum from the animals is pooled, reproducibility from lot to lot is clearly difficult. With the invention of monoclonal antibodies by Kohler and Milstein (1975), this all changed. By fusing a single B-lymphocyte with a single plasma tumor cell, it is possible to produce monoclonal antibodies in tissue culture. These antibodies can be selected for the desired specificity and affinity and, at least in principle, can be grown in large quantities (Yelton and Scharff, 1981). This invention has had a very large impact on research in several areas of biology, and the practical impact on clinical immunology will be very significant. If monoclonal antibodies can be made sufficiently inexpensive, they may even find industrial use, e.g., in purifying expensive chemicals.

The main problem in an immune test is to determine whether or not a reaction takes place when an antibody is introduced into a mixture. Since the outcome has great economic and medical consequences, many ingenious schemes have been invented for conducting these tests. One of them--the use of radioactively tagged molecules--has been quite successful commercially (Hunter, 1973). However, many radio-immunoassays may be replaced by tests in which molecules are tagged with enzymes, because of the potential hazards of using radioactivity (a rather small hazard in this case) (Blake and Gould, 1984). In addition to both these rather sophisticated tests, there are many simple tests, such as those based on agglutination (Weir and Herbert, 1973), on fluorescence (Weir et al., 1973), or on other optical effects (Giaever et al., 1984).

Several attempts have been made to develop an ideal immunological sensor that would transduce the immunological reaction directly into an electrical signal in real time (Lowe, 1984). For example, it is possible to couple the reacting molecules directly to the gate of a metal oxide semiconducting field-effect transistor (MOSFET) in an attempt to induce an immune reaction that will change the charge on the surface of the semiconductor sufficiently to produce a signal. To date, however, no successful immunotransistor has emerged, possibly

because its complex electrochemistry precludes reproducibility (Schenck, 1978).

MEMBRANE PROTEINS

The study of membrane proteins also offers possibilities for the development of biosensors. The outside surface of all living organisms and most organelles is separated from the interior by amphipathic lipid molecules that spontaneously form bilayers, or membranes, which are regarded as a two-dimensional liquid. These membranes contain important chemicals or molecules used by cells to carry out life processes, and serve as a solvent layer for the hydrophobic portion of membrane proteins, which are used by the cell to communicate with its surrounding environment and typically constitute half the dry weight of the membrane.

There are many different kinds of membrane proteins. Some are known to affect transport, signaling, catalysis, or other processes, but the functions of many others have not yet been identified (Singer and Nicholson, 1972).

A much studied membrane protein with potential application as a biosensor is bacteriorhodopsin. Because this protein forms two-dimensional crystals in the halobacteria, we know its three-dimensional structure to a resolution of 10A. When bacteriorhodopsin is activated by light, two hydrogen ions are transferred from the inside to the outside of the cell. We also know that the molecule consists of seven closely packed alpha-helices that span the membrane, but the detailed transport mechanism for the protons is still a mystery (Stockenius et al., 1979). The efficiency with which electromagnetic radiation is converted to chemical energy is approximately 2%. Although this is not very high, it is large enough to be of interest, certainly as a model system for biosensors.

The photoreceptors of vertebrates fall into two classes: the cone cells for color vision and the rod cells for monochromatic vision. The rod cells are the best understood: a membrane protein molecule called rhodopsin is responsible for transducing light into a chemical signal. This molecule carries the same prosthetic group as bacteriorhodopsin, but despite the similar nomenclature, that appears to be their only common feature. The mechanism of vision is not fully understood and is an extremely interesting scientific topic (Zurer, 1983). However, because there are several physical methods for detecting single photons--an important consideration in the development of biosensors--the photoreceptors of animals are only one of many possible subjects of research on the detection of light.

Several membrane proteins can transduce a chemical signal into an electrical signal. One such protein, the acetylcholine receptor of skeletal muscle cells, is a pentamer with a molecular weight of 250,000. This protein forms a doughnut-like structure or channel

in the membrane, which is generally called a ligand-gated channel because it opens and closes when acetylcholine binds to the receptor (Changeux, 1980). Such ligand-gated channels are an almost ideal mechanism for use as a biosensor. If we could produce gated channels that were sensitive to a specified ligand, any chemical signal could be converted to an electrical signal. But this cannot yet be done.

ENZYMES

Enzymes may provide superb biosensors and biomaterials, making further study in this area valuable. In living systems, virtually all metabolic reactions have an energy barrier that prevents their spontaneous occurrence. Such barriers are overcome individually by specific enzyme catalysts, which also often serve a regulatory role. Many thousands of enzymes are known, and probably orders of magnitude more are yet to be identified and characterized. As biocatalysts, enzymes are of central importance in the biosynthesis of biomaterials both in nature and in the laboratory.

Enzymes also play key roles in the functioning of biological receptors in vivo. Furthermore, any enzyme may in principle be used as part of a biosensor. The capability of enzymes in this regard depends on their specificity and affinity for binding the relevant compound (e.g., substrate or coenzyme) and also upon the amplification inherent to a catalytic system. In practice, the ability to quantify the reaction is also essential. This can be achieved in different ways: by the disappearance of substrate, by the appearance of a product or heat, or by a conformational change in the enzyme (Bergmeyer, 1983).

The utilization of enzymes for the production of biomaterials and for biosensors depends in large measure on an understanding of their basic properties. Indeed, specific perceived applications of an enzyme or enzyme system may not be readily pursuable because of inadequate fundamental knowledge concerning the enzyme. However, studies concerned with the use of enzymes for the detection of specific substances have been considerably stimulated by projected applications, many of which are already in use in clinical assays (Kricka and Carter, 1982). In these and other applications, enzymes are immobilized by attaching them to some solid support or matrix (Klibanov, 1983; Wienhausen and DeLuca, 1982). Such enzymes may have greater stability than free enzymes and can be more readily recovered for reuse--an important feature if the enzyme is costly. In more complicated systems involving a pathway with many sequential enzyme reactions, all enzymes immobilized together on the same matrix are remarkably more effective than homogeneous systems--systems with all participating species in solution--in increasing overall rates. A reason for this may be that the product of the first reaction is closer to the second enzyme in the pathway, and so on down the line. Pathways with as many as 12 enzymes have been tested to study this phenomenon (DeLuca and Kricka, 1983).

The stability of enzymes is crucial to their utility in various applications. Therefore, basic knowledge concerning the fundamental chemistry and properties related to enzyme (protein) stability must be amassed. Of importance are studies of organisms from extreme environments, such as the recently described organisms from deep-sea vents, which are able to survive and grow at temperatures well above 100°C, and organisms that reside in hot springs. Because thermal stability is a key consideration, knowledge of thermophilic organisms, including their proteins and enzymes (and their nucleic acids), should be of great value.

Chapter 5

POLYSACCHARIDES

Polysaccharides are polymers of the large group of compounds and materials made up from a relatively extensive list of monosaccharides, which are known to consist of 3-, 4-, 5-, 6-, or 7-carbon compounds. All the higher sugars are related to D-glyceraldehyde, which in higher sugars is represented by the asymmetric carbon atom most distant from the functional group. Many of the hydroxyl groups may be modified by replacing them with amino groups or by other means, such as oxidation of either terminal carbon atom to give uronic or other acids, which constitute another class of monomers.

The earliest known, the largest, and the most common groups of polysaccharides are cellulose and starch (Aspinall, 1970, 1983). In general, these have been perceived as molecules having repetitive structures with little variation. Cellulose is a polyglucose linked at the 1,4 position in the beta configuration at the 1-carbon atom, whereas starch and all its congeners are linked primarily at the 1,4 positions at the anomeric 1-carbon atom, which is in the alpha configuration.

A listing of the fragments of starch, such as amylose, demonstrates the simple linear arrangement of both starch and cellulose. However, it also shows the enormous possibilities for variation (Brant, 1980; Burton and Brant, 1983). Substitution and branching lead to an enormous variety of structures, even greater than for amino acids or nucleotides.

IMPORTANCE OF POLYSACCHARIDES

An important aspect of polysaccharides is the recent recognition of their enormous variety of structures--not only because they consist of more than a dozen different monomers but also because each monomer has many points of linkage in the polymer--an effect not true of either nucleic acids or proteins (Brant, 1980). Polysaccharides were initially a subject of interest because of their ubiquity as the major structural material of plants and a major energy-storing material of both plants and animals. It is now becoming apparent, however, that polysaccharides have many more functions than those two simple ones

(McNeil et al., 1984). The structural variety of the polysaccharides found on the surfaces of cells may help cells recognize each other with high specificity. This suggests the potential importance of polysaccharides in the development of biosensors and biomaterials. In fact, at least one such biosensor has already been described (Mansouri and Schultz, 1984).

Furthermore, highly valuable products may be derived from polysaccharides at specific points. This could lead to the development of critical industrial materials, such as thickening agents, gels, and a variety of adhesives (Sandford and Baird, 1983).

SPECIFICITY OF POLYSACCHARIDES

Polysaccharides have long been known to be the determinants of the blood group characteristics A, B, O, and Rh (see Aspinall, 1970). The cell surface polysaccharides are now known to be specific to all kinds of animal cells as well as bacteria (Aspinall, 1983). Some bacterial polysaccharides can be prepared in large amounts and can be used as biomaterials. Mansouri and Schultz (1984) have described the use of the specificity of a particular polysaccharide in the development of a biosensor. In their example, the recognition of glucose and its polysaccharide dextran by the glycoprotein concanavalin A has been used to devise a microsensor for the continuous determination of glucose in a complex mixture by optical measurement. This generic device could be adapted to the determination of any material for which a specifically binding polysaccharide receptor could be found or made.

POLYSACCHARIDES AS BIOMATERIALS AND BIOSENSORS

Many polysaccharides, both natural and modified, have been applied in a variety of industries (Sandford and Baird, 1983). Their adaptability is due not only to the enormous variety of their structures and properties but also to their amenability to modifications. Table 5-1 lists some of these applications, with emphasis on microbial polysaccharides (Sandford et al., 1984). The specific nature of each application depends markedly upon the properties given to the polysaccharide by its structure, which is not known in all cases. In general, properties of polysaccharides are those of the macromolecules that can interact with the solvent and with other materials that they contact, thus conferring stabilizing, thickening, and adhesive properties and other characteristics (Sandford and Baird, 1983).

PROBLEMS AND OPPORTUNITIES

Clearly, the major need in the further development of polysaccharides as biosensors, biomaterials, or any other use is a detailed

Table 5-1. Some Applications of Polysaccharides

Applications	Polysaccharides	Properties
Adhesives (also see Paper)		
Latex	Cellulose derivatives	Viscosity
Tile mortars (cement)	Methyl cellulose	Hygroscopicity, viscosity
Wallpaper	Algin, starch, modified starch	Thickening ability
Agriculture		
Flowable pesticides	Xanthan gum	Suspension-drift control
Liquid fertilizers	Xanthan gum	Colloidality
Liquid feed supplements	Xanthan gum, guar gum	Colloidality
Ceramic, Refractories, Welding Rods	Algin	Slipperiness
	Xanthan gum	Colloidality
Cleaners, Polishes	Xanthan gum	Abrasiveness, colloidality, acid and base stability
Detergents	Carboxymethyl cellulose	Emulsifying, wetting, cleaning ability, colloidality
Explosives		
Ammonium nitrate slurries	Guar gum, hydroxypropyl guar gum, xanthan gum	Water resistance
Package gels	Guar gum, hydroxypropyl guar gum, xanthan gum	Calcium nitrate compatibility
Fire-Fighting	Guar gum, guar derivatives, xanthan gum	Foam stabilization ignition retardation
Ink (Flexo, Gravure, Jet) Lithography	Gum arabic	Viscosity
Metal-Working		
Refractory coatings	Gum arabic	Colloidality
Mining (Heavy Media Separation)	Xanthan gum, starch, guar gum	Colloidality
Oil Fields		
Drilling muds	Xanthan gum, cellulose ethers	Viscosity, colloidality
Enhanced oil recovery by polymer flooding	Xanthan gum	Viscosity
Desiccators	CMC	Hygroscopicity
Hydraulic fracturing	Hydroxypropyl guar gum, hydroxyethyl cellulose, xanthan gum, carboxymethyl cellulose	Colloidality, hygroscopicity, viscosity reduction by chemicals and enzymes, cross-linking ability
Acidizing	Xanthan gum	Colloidality, stability in the presence of strong acids at elevated temperatures

Table 5-1. Continued

Applications	Polysaccharides	Properties
Paint		
Commercial latex, maintenance coatings, industrial coatings	Hydroxyethyl cellulose and methyl cellulose (microbial biopolymers)	Viscosity, colloidality
Paper		
Coatings	Algin, carboxymethyl cellulose	Viscosity
Sizing agents	Starch, modified starch, algin	Film-formation, hygroscopicity
Particle board, corrugated board	Starch, modified starch, algin	Glue extender
Photography	Sodium cellulose sulfate	Antistatic coating extender
Polymerization		
Emulsion	Hydroxyethyl cellulose	Protective colloidality
Suspension agent	Xanthan gum	Colloidality
Room Deodorant Gels	Carrageenan	Gel stabilizer
Textiles		
Warp sizing	Starch, modified starch, CMC	Film-forming ability
Printing and dying (retards dye dispersion)	Algin	Viscosity
Pigment printing	Modified starch, hydroxyethyl cellulose, methyl cellulose, hydroxypropyl guar gum, guar, locust bean gum	Viscosity, binding compatability
Jet printing	Modified starch, hydroxyethyl cellulose, methyl cellulose, hydroxypropyl guar gum, guar, locust bean gum	Viscosity
Fabric finish	Cellulose	Fiber substantivity

knowledge of their structure, which is much more complex in principle than that of the other two biopolymers (proteins and nucleic acids). This complexity is attributable primarily to the multifunctional character of the monomers that constitute the polysaccharides and the ease with which those functions can be modified, as well as to the variety of ways in which the polymer can be constructed because of the multifunctional character of the monomer. However, the first principal requirement is a detailed knowledge of the fine structure of the polymer itself, not merely the nature of the monomers of which it is constructed, their derivatives, and the modes by which they are linked together.

The determination of this structure has been made possible only through technical innovations that have become available in the last decade or so. The first of these technologies involves the new physical methods for examining the structure of large molecules. These include nuclear magnetic resonance (NMR), both proton and carbon spectroscopy in all their forms, such as Fourier transform spectroscopy, time-resolved spectroscopy, and decoupling technology for different nuclei. The second technology, also applicable to polymers as a whole, is high-resolution Fourier transform infrared spectroscopy, which provides information not only on the nature of the monomers that are present but also, in some cases, on the secondary and tertiary structure as well. The third is circular dichroism and magnetic circular dichroism as additional techniques for structure determination as well as mass spectroscopy of the original polysaccharide, its derivatives, and its pyrolysis products.

Following examination of the intact polymers by the methods just described, it is expected that the polymers will be fragmented in as specific a manner as possible. The generally hydrolytic breakdown of the polymer into random fragments has been and still is an important technique, but better yet will be the development of specific methods for breaking the polymer at specific points in its structure, whether it be a linear structure or a branching point. If such a purely synthetic chemical means can be developed, other than the randomly discovered enzymes that might break the polymer at some specific point selected by the researcher, it would be on enormous step forward. Such methods are beginning to appear in this field as well as in others, and further development will probably be forthcoming as the needs become more apparent.

Once the unmodifed polymer has been broken into smaller fragments, which are more amenable to detailed structure determination by physical and chemical methods and derivatization followed by physical and chemical examination, detailed linear and branching structure can ultimately be determined. The development of new methods for determining structure would represent significant progress in the use of polysaccharides in both biosensors and biomaterials because they are enormously complex. There is also a need to know much more about the detailed three-dimensional structure of polysaccharide molecules, as well as their fiber and film structures, before we can make use of them in ways that have been suggested and perhaps others that have not yet been imagined.

Thus, there is an opportunity to develop new instrumentation and new technologies for determining the structure of polysaccharides. This should result in a better understanding of many developmental problems in biology that have their origin in cell recognition and lead to the development of biosensors and biomaterials based on the same principles.

Chapter 6

SYNTHETIC ANALOGS

Molecules derived from living sources have special properties and perform very specific functions in the biological organisms from which they are obtained. As modern analytical capabilities have increased, many of these properties have been explained by laws of chemistry and physics. A long-standing goal of chemists has been the duplication of properties and functions of complex molecules derived from living systems--biomolecules--through synthesis. Hence, the field of synthetic analogs and systems has been an integral part of organic chemistry for decades.

Among the terms used at various times to describe these endeavors and products are biomimetic chemistry (Breslow, 1972), bioorganic chemistry, synthetic enzymes, synzymes, host-guest chemistry (Cram and Cram, 1978), and artificial biocatalysts. To date, most studies on synthetic analogs have dealt with enzymes (Breslow, 1979, 1982; Maugh, 1984a,b; Rebek, 1984); others have focused on hormones (Kaiser and Kezdy, 1984), artificial membranes (Aizawa, 1985; Pusch and Walch, 1982), and photoconversion systems (Bolton, 1983; Calvin, 1983).

There are several major reasons for preparing and using synthetic analogs instead of their biological counterparts: their stability; ease of synthesis; isolation and purification; modification and derivitization; their specific activity; their role in fundamental research; and their potential for using different reaction pathways.

- Stability. Synthetic molecules or systems may be designed to have greater stability than biomolecules during operation or simply in storage. Desirable qualities include stability at extremes of temperature and pH, in organic solvents, in solvents of high ionic strength, and in oxidizing media. Although biological molecules operate predominantly in an aqueous environment and at ambient temperatures, most traditional organic chemistry has been conducted in nonaqueous solvent systems.

- Ease of synthesis. Small synthetic analogs and systems are often easier to synthesize on a large scale than are their larger biologically

produced counterparts. Consequently the advances in solid-phase peptide and oligonucleotide syntheses favor the preparation of these compounds by traditional approaches. Our knowledge of complex biological systems, and thus our ability to construct the desired analogs, is less. This hinders our ability to use classical organic chemistry. In this case, hybrid syntheses--using biological as well as synthetic chemical techniques--are preferred. For example, cyclodextrin molecules used as model enzymes can be produced by either microbial or enzymatic means. The basic molecule is synthesized more easily by biological than by chemical means; however, once the molecule has been synthesized, chemical modifications are almost routine.

With the advent of recombinant DNA technology, large scale synthesis of proteins and enzymes became easier, and many previously unobtainable proteins and hormone peptides have been produced in gram quantities through genetic engineering. However, the current capabilities in genetic engineering rely to a large extent on chemical synthesis technology--namely, sequencing and synthesis of nucleic acids. The pure chemical synthesis of a few enzymes and genes was achieved more than a decade ago.

- Isolation and purification. Once the desired compound is produced either chemically or biologically, it must be isolated and purified. Here again, synthetic systems have decided advantages over biological ones in that the production process can be designed to achieve maximum yield with appropriate isolations being chosen from several options. Isolation of the desired compound from the milieu of hundreds of other compounds can be troublesome and extremely costly. Even with recombinant DNA-produced insulin, "downstream processing" is the major cost associated with the process.

- Modification and derivatization. Well-designed synthetic analogs can be further modified to produce a range of compounds with a variety of properties. Of course, some resulting features may be unexpected, and the modified analog could be even more effective than initially envisioned. Moreover, because synthetic analogs are more stable and their molecular weights are usually lower than those of their biological counterparts, it may be possible to use them in the development of drug delivery systems that are far more effective than those derived from biological molecules.

- Specific activity. Large macromolecules of biological origin may perform their function well, but since only a small part of the molecule exerts the desired effect, large quantities may be needed. For example, the catalytic site on enzymes is usually only a small part of the total molecule. Thus, a large portion of an enzyme is not involved in catalysis, but is devoted to other functions such as binding to membranes and preventing the degradation of the molecule by other enzymes. Nevertheless, on a weight basis, small synthetic analogs could have a higher specific activity than large biological molecules.

o Role in fundamental research. An important but often unappreciated role of synthetic analogs and systems is their use in obtaining fundamental knowledge about chemistry and life processes. With synthetic analogs, individual steps in complex reactions can be isolated, fine details of kinetic and structural features can be examined, and in general a greater understanding of biological systems can be achieved.

o Different reaction pathways. In addition to duplicating exact chemical reactions in biological counterparts, synthetic analogs could be designed to produce the same desired product through a different reaction pathway. Hence, synthetic analogs not only mimic biological reactions but they might be used to achieve synthesis under different operating conditions.

CURRENT RESEARCH

For the past three decades, research on synthetic analogs has been focused on enzymatic catalysis, that is, biocatalysis. Despite recent advances, it is still largely an empirical science, and the design of effective catalysts and synthetic analogs continues to be an important and somewhat elusive goal. The achievement of binding and rate acceleration are key indicators of success. Yet even when high rate accelerations and rate constants close to those of natural enzymes are attained, selectivity may be poor. On the other hand, selectivity may be high but rate acceleration may not be great. It is also important to be sure that increased rates of reaction are due to true catalytic turnover on the part of the mimic rather than merely to its role in transformation.

Interest in the properties and production of enzymes and proteins altered by site-specific mutagenesis is called protein engineering (Ulmer, 1983). Several additional approaches to the preparation of enzyme analogs are discussed below.

Breslow (1979, 1982) has developed synthetic analogs based on cavity-forming sugar molecules called cyclodextrins. The substances are modified chemically in a variety of ways to give analogs that mimic a variety of natural catalysts. Cyclodextrins are composed of six to eight glucose residues that have hydrophobic interiors and hydrophilic exteriors. Thus, the cyclodextrins have the ability to extract small organic molecules from a water solution and bind them into cavities. This is similar to the ability of enzymes to bind substrates into their interior cavities.

Cyclodextrin binding is selective for molecules with a specific shape. Several chemical transformations have been examined, and enzyme-like behavior has been observed. For example, a cyclodextrin-based analog, bisimidazole compound 1, has two catalytic imidazole groups that imitate the enzyme ribonuclease in hydrolyzing phosphate ester bonds (see Figure 6-1). The binding site in compound 1 in the figure is not well suited for binding the macromolecule RNA. In the presence of the analog, substrate 2 is hydrolyzed selectively only

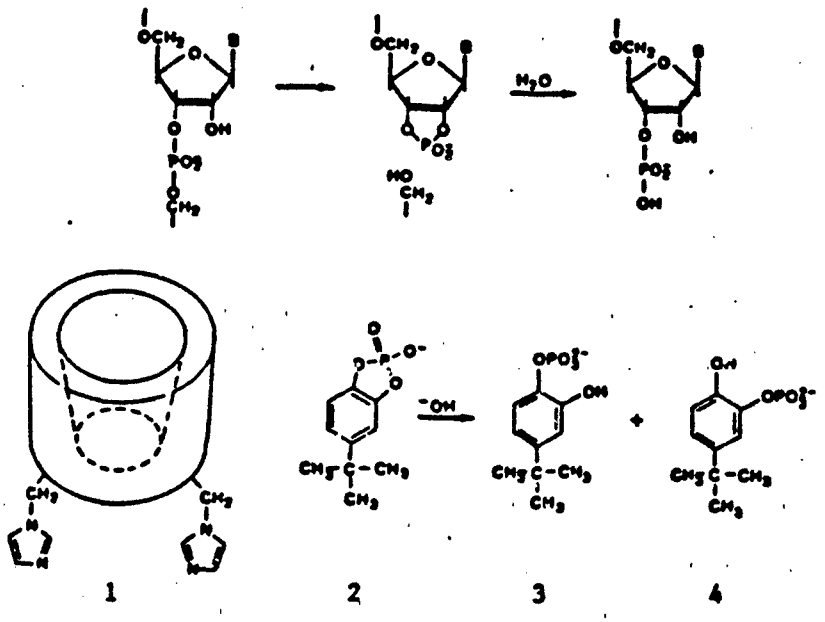

Figure 6-1. The sequence by which ribonuclease hydrolyzes RNA (top). Compound 1 is a cyclodextrin bisimidazole artificial enzyme that catalyzes the hydrolysis of substrate 2 by an enzyme-like mechanism. Under simple hydrolysis, 2 yields a mixture of 3 and 4. From Breslow, 1982, with permission from Science.

the presence of the analog, substrate 2 is hydrolyzed selectively only to compound 3—not to the isomeric mixture of 3 and 4, which is achieved under chemical hydrolysis. Selectivity in this case is achieved by the geometry of the catalyst-substrate complex. Compound 1 mimics the second step in the reaction of ribonuclease with RNA.

Compounds that produce enormous rate increases in reactions have been synthesized. The most striking rate accelerations have occurred with substrates based on a ferrocene nucleus that fits beta- cyclodextrin well and is strongly bound into the cavity (Breslow, 1979, 1982). The attack by a hydroxyl group within the complex was 750,000 times as fast as a simple attack by a solvent under the same conditions. In another example, rates increased 6 million times with one of the two mirror-image isomers of the substrate in the ratio of 65 to 1. The reaction was performed in a mixed organic-water solvent.

Another approach being advanced principally by Cram and colleagues depends on totally synthetic compounds called crown ethers or "chorands,"

which have interior dimensions like those of natural proteins. In addition to chorands, which can fold like sacks to fill their own cavities, there are molecules called cryptands, whose parts can rotate to fill their own cavities, and spherands, which contain spherical cavities lined with unshared electron pairs (Cram and Cram, 1978; Metzger, 1983) (Figure 6-2). The general term cavitands applies to organic molecules deliberately constructed to contain cavities of varying sizes and depths. In a recent review of supramolecular chemistry, i.e., the study of structures and functions of supermolecules that result from binding substrates to molecular receptors, Lehn (1985) provides further examples of directed coordination phenomena that affect molecular recognition, catalysis, and transport. These include tetrahedral recognition by macrotricyclic cryptands, anion receptor molecules, macrocyclic receptors for ammonium ions, metalloreceptors, and supramolecular catalysts.

It is also useful to modify the properties and hence function of enzymes through chemical means. One example is the work of Kaiser and his colleagues (Slama et al., 1984), who transformed the proteolytic enzyme papain into an oxidoreductase by attaching a synthetic flavin to it. The new semisynthetic enzyme accelerates the rate of oxidation of dihydronicotinamides two to three orders of magnitude over the rate of nonenzymatic oxidations.

Another recent example is the work of Saraswathi and Keyes (1984), who modified bovine pancreatic ribonuclease by exposing it to acidic conditions in the presence of indole propionic acid and the crosslinking agent glutaraldehyde. The new enzyme thus generated, an acid esterase, exhibited esterase activity with a number of common ester substrates, whereas no esterase activity was detected in the natural enzyme. The new esterase possessed a broad specificity, but there was a preference for amino acids containing an aromatic group. This approach has also been used by the same investigators to prepare other semisynthetic enzymes, including beta-glucosidase from alpha-amylase, an esterase from bovine serum albumin, and an alpha-chymotrypsin-like catalyst from trypsin.

Enzymes with new properties can also be created by site-specific modification--an approach pioneered nearly 20 years ago. The dramatic modification was the alteration of a serine residue at the active site of the proteolytic enzyme subtilisin to produce a sulfhydryl group. The resultant thiol subtilisin, in which sulfur replaced the oxygen, did not possess proteinase activity and was reactive with only very activated substrates such as p-nitrophenyl esters (Maugh, 1984b). Recombinant DNA techniques introduced very recently modify proteins and enzymes at specific sites in the hope of modifying their properties--the so-called site-directed mutagenesis approach (Maugh, 1984b). In one technique, the gene that codes for the protein is cloned and incorporated into a suitable carrier such as a plasmid or bacteriophage. Then, an oligodeoxynucleotide primer containing 15 to

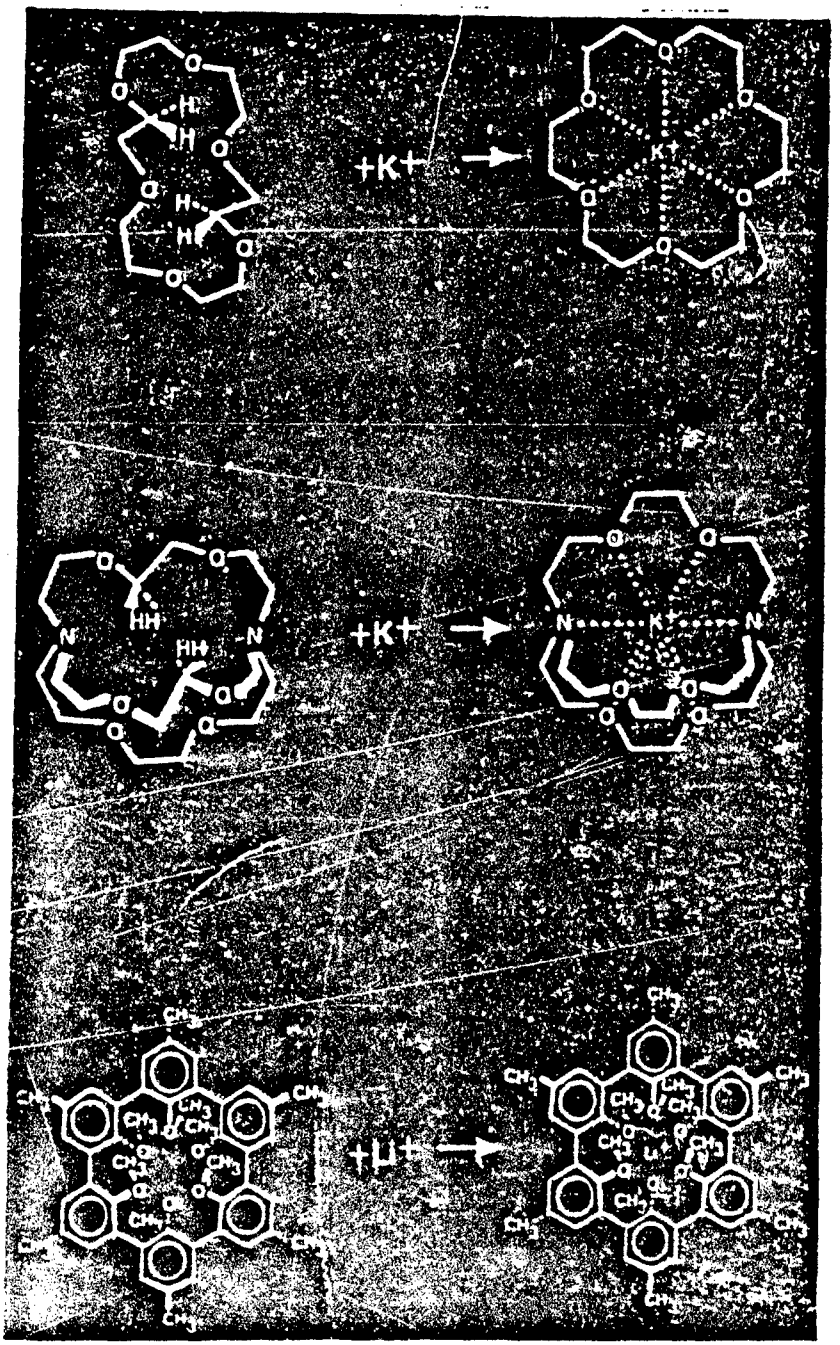

Figure 6-2. Chorands (1), like sacks, can fold to fill their own cavities. The different parts of cryptands (2) can rotate to fill their own cavities. Spherands (3) contain cavities lined with unshared electron pairs.

20 nucleotides is synthesized with the desired mutation, typically a change of only one deoxynucleotide. The sequence retains sufficient homology for hybridization with the host DNA, and DNA polymerases then use the primer to synthesize a complementary copy of the plasmid or vector. The copy is separated from the original and is used to control the production of the mutant in an appropriate host.

An alternative methodology involves the enzymatic removal of a segment of the cloned natural gene containing the site where mutagenesis is desired and replacement with a segment containing the desired change. Fersht et al. (1985) used this site-specific mutagenesis approach in investigating the reaction kinetics of tyrosyl-tRNA synthetase.

Protein engineering and site-specific mutagenesis offer a more rational approach to producing superior enzymes and proteins. However, no synthetic analogs of enzymes or modified enzymes are being used commercially at present.

RELEVANCE TO BIOSENSORS AND BIOMATERIALS

Although synthetic analogs and systems, especially synthetic enzymes, may not possess all the desired properties of their natural counterparts, their potential use in sensing devices and biomaterials is very appealing. Especially desirable features of synthetic analogs would be enhanced stability at higher temperatures and in extreme environments, their superior ability to be fabricated into devices due to a higher net effective concentration of active species, and the relative ease with which they can be synthesized.

The use of synthetic analogs as biosensors is limited by their low selectivity or affinity for the counter molecule of interest. This may be ameliorated by the results of current efforts to design new host molecules and synthetic enzymes with superior properties.

Many novel uses of biomaterials can be contemplated. For example, synthetic analogs of enzymes such as cyclodextrins may be useful in industrial separation processes (Wernick and Scypinski, 1984). In addition, there is a wide array of possible uses for special materials, ranging from pharmaceuticals to polymers with unusual properties.

The semiconductor industry has very successfully reduced the size of circuit elements to the extent that a transistor is not much larger than a biomolecule. Many attempts have been made to couple the integrated circuit technology directly to some form of a biosensor, but without much success. This work is very promising for synthetic analogs, however, since biomolecules generally lack the stability for industrial use. Several polymers such as polypyrrole can be switched repeatedly from a conductive state to an insulating state by oxidation

and reduction and in principle offer promise as biosensors. Because such devices depend on chemical reactions, the response time will be limited by diffusion, and much can be gained from making the device physically small by borrowing technology from the semiconductor industry (White et al., 1984).

Ion-selective membranes or electrodes are also often used. These allow electrical measurement of the product or substrate from a nearby immobilized enzyme. A few sensors of this type have reached the commercial market (Lowe, 1984).

Chapter 7

HOLISTIC FUNCTIONS OF MARINE ORGANISMS

Marine and estuarine environments are characterized by their salinity, temperature, buoyancy, nutrient concentrations, dissolved oxygen, turbidity, and hydrostatic pressure. The biota of the oceans must therefore adapt to the diverse conditions of their environment, which may often be rigorous. In some instances, marine organisms may have special adaptations, such as in symbioses (Cavanaugh, 1983; Cavanaugh et al., 1981; Felbeck, 1981, 1983); adherance to surfaces, which occurs, for example, in biofouling and in communication; and chemosensory signal transduction (Goulbourne and Greenberg, 1981, 1983a,b; Kathariou and Greenberg, 1983). Exploration of the genetic bases for these adaptations, as well as detailed investigation of the phenomena themselves, may prove to be of great value for biotechnology, if novel proteins, carbohydrates, and nucleic acid structures are found. Organisms living near or in hydrothermal vents probably have thermostable or thermophilic enzymes (Baross and Deming, 1983; Baross et al., 1982, 1984; Deming, 1984), which may be one example of a novel system.

Marine biology has not been the focus of intensive research support as have, for example, the health-related fields or elementary particle physics. As a consequence, it remains a largely descriptive science. However, recent discoveries and technological advances provide unique opportunities for studying the marine environment. The potential for basic research in this area by the Navy should lead to developments in marine biotechnology of value in naval applications (Colwell, 1983). Of particular promise is research in deep-sea biology, the archaeobacteria, marine plasmids, marine fishes, marine plants, and biofouling.

DEEP-SEA BIOLOGY

The Challenger Expedition of 1873-1876 is often credited as the beginning of deep-sea biology. Since that time interest and activity in marine biology have grown. The finding of live specimens at great depths negated the azoic zone theory that no life existed below 600 m, which was suggested in about 1840 by Edward Forbes, a British expert

in oceanography. During the ensuing years, living organisms, including bacteria, animals, and other life forms, have been recovered from ocean depths greater than 1,000 m. Recently, barophilic organisms have been isolated from an amphipod collected in the Mariana Trench at a 10,500-m depth (Yayanos et al., 1981) and from other deep-sea sources (Deming et al., 1984). Earlier discoveries of unusual marine animals and microorganisms and the beginnings of marine microbiology have been documented by Benecke (1933) and ZoBell (1946).

Dense and thriving populations of invertebrates were discovered around the hydrothermal vents at depths of approximately 2,600 m (Ballard, 1977; Corliss et al., 1979; Lonsdale, 1977). Results of several studies suggested that various kinds of bacteria, taking the place of photosynthetic primary producers of organic carbon, use reduced inorganic constituents of the emitted hydrothermic fluid at the vents as a source of geothermal energy for chemosynthesis (Cavanaugh, 1983; Cavanaugh et al., 1981; Felbeck, 1981, 1983; Jannasch and Taylor, 1984; Jannasch and Wirsen, 1979).

It has been established with reasonable certainty that the major and most efficient transfer of chemosynthetically produced organic carbon to the vent invertebrates takes place by a newly discovered type of symbiosis (Cavanaugh, 1983; Cavanaugh et al., 1981, Felbeck, 1981; 1983). There is no evidence that the densely clustered populations at the vents receive a substantial food supply from an organic source (Jannasch and Taylor, 1984). These unusual events observed at the thermal vents have renewed appreciation of symbiosis and its role in natural ecosystems.

Tube worms at the vents are mouthless and gutless and have in their body cavity an organ filled with spongy tissue called the trophosome, which contains procaryotic cells (Cavanaugh et al., 1981). Chemosynthetic symbiosis is responsible for a large part of the nutrition of these animals (Southward, 1982; Southward et al., 1981).

Interestingly, sulfates are reduced geothermally in the vents. The hydrogen sulfide released in the vent fluid is taken up by the bloodstream of the animals along with oxygen from the seawater.

Recent explorations at 2,650-m depths along the East Pacific Rise have revealed spectacular 3- to 17-m high sulfide chimneys or "black smokers" scattered along axes of seafloor spreading centers (Jannasch and Taylor, 1984; Spiess et al., 1980). Spewing from these submarine vents into the surrounding cold seawater are jets of hydrothermal fluid at temperatures that often exceed 350°C. (Hydrostatic pressures at the vents retain seawater in the liquid phase up to 460°C.) These fluids contain supersaturated concentrations of reduced gases and metals, providing unusual geothermal sources of inorganic energy for chemosynthetic bacteria flourishing at

temperatures of 2°C to 40°C on surfaces and in seawater surrounding the vents (Karl et al., 1980). The oxidation of hydrogen sulfide is the source of energy used to create bacterial biomass.

Baross et al. (1982) reported the culture of viable marine thermophilic microorganisms at temperatures as high as 306°C--more than 100° higher than previously successful culture temperatures. Subsequently, cells have been reported to be culturable under controlled laboratory conditions at a pressure of 265 atm and temperatures of at least 250°C, simulating vent conditions from which the cultures were isolated. If such reports are confirmed, these extremely thermophilic microorganisms from submarine volcanic vents offer great potential for biotechnology. However, advances in this area will be dependent on the acquisition of an increased understanding of the growth, metabolism, and genetic characteristics of these microorganisms (Deming, 1984).

THE ARCHAEOBACTERIA

In parallel with the deep-sea discoveries, Woese and his colleagues proposed that a large group of unusual organisms--the archaeobacteria--have a common phylogenetic origin (Kandler, 1982; Woese and Fox, 1977). This proposal followed their analysis of the sequences of oligonucleotides obtained from the 16S ribosomal RNA of a large number of bacteria by hydrolysis with endonuclease. The nucleotide sequences were compared by using a binary association coefficient (Fox et al., 1977). According to Bergey's Manual of Systematic Bacteriology (Krieg and Holt, 1984), the archaeobacteria comprise three metabolically diverse groups: the strictly anaerobic methanogenic bacteria, the obligate halophiles, and the thermoacidophilic group (Thermoplasma, Sulfolobus, and the Thermoproteales).

The possibilities for using archaeobacteria in biotechnological applications have not been ignored (Kandler, 1984). The unusual lipids, nucleic acid and genome organization, and metabolic functions of these organisms offer great opportunities for advances in biotechnology.

PLASMIDS IN MARINE BACTERIA

Plasmids are widely distributed among marine bacteria in the deep sea (Olson et al., 1978) and in the Antarctic (Kobori et al., 1984). Much of the literature about them suggests that extrachromosomal elements are possible agents of adaptation and development (Reanney, 1976). Obviously, they may play a major role in the marine environment as well. The genetics of marine bacteria is beginning to be elucidated (MacDonell and Colwell, 1984 and in press). Shuttle cloning vectors have been described (Datta et al., 1984), and genes have been cloned (Potrikus et al., 1984; Wortman et al., 1985).

MARINE FISHES

Recently, genetic engineering has been applied to fish (Takeshita et al., in press). A specific example of genetic manipulation in marine fishes involves the antifreeze gene complex (Gourlie et al., 1984). These genes are regulated by changes in environmental temperature. The antifreeze genes can be used as a model of the ways that environmental temperatures affect functions of higher organisms. They also have a potential practical application in the storage of organs and cells at low temperature.

Specifically, the antifreeze genes are involved in the synthesis of polypeptides that contain periodic saccharides. These antifreeze proteins depress the freezing point of fish fluids (DeVries, 1971, 1984). Both antarctic and arctic fishes, which continually experience low temperatures, have antifreeze genes that are always turned on. However, fishes living in temperate regions experience cold water seasonally and need antifreeze proteins only in the cold seasons. For example, these proteins are detected in the winter flounder (Pseudopleuronectes americanus) only during the cold months when temperatures can drop to 1.5°C from summer temperatures as high as 20°C. In vivo synthesis of these proteins has been correlated with changes in photoperiod as well as with temperature shifts (Duman and Devries, 1974). During winter and spring (November to April), few proteins other than antifreeze are synthesized. In summer, no significant antifreeze mRNA can be detected. The appearance and disappearance of this mRNA are correlated with seasonal changes in antifreeze protein in the serum (Gourlie et al., 1984).

Antifreeze proteins have been sequenced (DeVries, 1984). For the winter flounder, the DNA nucleotide sequence has been determined as well (Gourlie et al., 1984). In some cases, distantly related fishes have similar antifreeze proteins, whereas some more closely related fishes have quite different ones (DeVries, 1984). In the winter flounder, there is a family of antifreeze genes (Gourlie et al., 1984). Studies of the structure of the antifreeze proteins of this fish indicate that the polar residues aspartate and threonine are spaced in such a way that they may interrupt the ice lattice, thereby depressing the freezing point (DeVries, 1984). The antifreeze system is an excellent model for studying environmental factors affecting gene expression and associated cellular events in marine organisms.

MARINE PLANTS

DNA technology has recently led to the development of methods to regenerate plants through protoplast fusion. This procedure may circumvent barriers of conventional hybridization and result in the production of new varieties of plants. The development of economically feasible sources of renewable fuels from green plants, including marine algae, demonstrates that there is a considerable

potential for the production of commercially important hydrocarbons that may be used as fuel oils.

Biotechnology has also resulted in improved quality and increased quantity of hydrocarbons produced by plants. Plant cell culture has been commonly used to induce somaclonal variation by taking advantage of preexisting tissue variation and mutation. In addition, protoplast technology enables investigators to transfer desirable genetic traits between oil-producing species and to regenerate plants through protoplast fusion.

Novel recombinant DNA methods are being developed to transfer genes between distantly related organisms. Gene transfer by somaclonal variation, protoplast fusion, or recombinant DNA can result in the production of annual crops yielding hydrocarbons or containing genes controlling characteristics involved in resistance to diseases, insects, and herbicides. New varieties have not yet been produced by protoplast fusion. In the future, however, it is expected that additional species will be regenerated from protoplasts and that there will be an emphasis on cytoplasmic traits and on agriculturally important crops and traits. Integration of DNA technology into breeding programs and interfacing it with other cell culture techniques could result in novel harvests if applied to marine algae and seaweed culture.

Plant chloroplasts may be useful vehicles for gene transfer and may provide a method for dealing with somaclonal variation, cytoplasmic male sterility, herbicide resistance, and photosynthetic efficiencies. Lysosomal fusion and molecular fingerprinting may also provide more concise characterization of new plant varieties. The methods discussed above have only just begun to be applied to marine plants. In addition to the production of fuels, the polysaccharides of marine algae, including agar and carrageenan, are products of economic importance as well as of scientific interest (Colwell, 1983).

BIOFOULING

Significant advances have been made in understanding the early stages of microbial film formation preceding attachment of macroorganisms to surfaces. Much of it has derived from research on interactions between procaryotes and invertebrates. A specific example is the association between a bacterium and the oyster Crassostrea virginica. The bacterium produces melanin and a viscous slime layer that facilitates strong adhesion, i.e., an exopolymer.

An unusually large variety of marine procaryotes (Hyphomonas spp.) were recovered from a single mussel-like animal in hydrothermal vents at a depth of 2,500 m near the Galapagos Islands. These bacteria also synthesize adhesion polymers and melanins, and they tolerate a wide range of environments, multiplying within a pH range of 5 to 10 and

in the presence of 2% to 18% salt in temperatures of 3°C to 50°C. They also grow well at atmospheric pressure and are far more resistant to pressure changes than are terrestrial procaryotes (Weiner et al., in press). Thus, species of Hyphomonas are one example of many organisms that are highly suitable as recombinant DNA hosts for expression of genes from terrestrial and estuarine organisms in the environment as well as for probing the genetic basis of attachment and production of adhesion-promoting metabolites.

Genetic engineering of marine organisms is moving forward very rapidly in a variety of applications similar to those where genetic engineering has already been successfully applied with land-based organisms, notably to control bacterial attachment in biofouling, to modify biopolymer surfaces, to reduce or eliminate the effects of toxins, and to recover metals. Biofouling involves a complex series of linked events, starting with adsorption of biopolymers to surfaces and bacterial adherence to the polymers, followed by colonization by other organisms on the conditioned surfaces. Clearly, research is needed to understand the basic biology, chemistry, and physics of these events, as well as the role of chemoreception in biofouling. One possibility for the control of biofouling would be modification of biopolymer surfaces so that bacteria cannot recognize them.

MARINE BIOSENSORS

In the mid-1970s Blakemore (1975) reported the startling discovery of magnetotactic bacteria--singular noncontroversial evidence that organisms can detect a magnetic field. For decades, a number of investigators have suggested that several organisms from birds to bees use magnetic fields for orientation and navigation, but this has never been proven. Because the earth's magnetic field is much too small compared to thermal noise to have an effect on isolated atoms at ordinary temperatures, it follows that organisms must possess a ferromagnet to respond to the field, as do the bacteria. Since the discovery of magnetotactic bacteria, there has been a flurry of reports of the presence of magnetite in higher organisms, but there is not enough evidence at present to relate such a presence directly to navigation.

Sharks can detect electric fields as small as 5 nV/cm (Kalmijn, 1981). This is surprising, because their detection ability may be hampered by a number of factors, including thermal noise, the electric fields generated by the shark itself, and the electric field generated by ocean currents resulting from the magnetic fields (Kalmijn, 1981). In fact, sharks may possess an internal signal-processing system that can discriminate between various electrical signals.

The 5nV/cm detectable by the shark would correspond roughly to 500 nV, if it had an antenna 1-m long. Johnson noise is given by $(4kTR\ \text{delta-f})^{1/2}$, where k is the Boltzmann constant, T is

temperature, R is resistance, and delta-f is the bandwidth. For a bandwidth of 10 Hz and a 1-megohm resistor, Johnson noise corresponds to approximately 500 nV at room temperature. Thus such detection by the shark is feasible. It is unfortunate that this work has not been corroborated, because such a sensing system probably would outperform any available artificial device.

Marine macro- and microorganisms offer a gene pool as yet untapped. Luminous marine bacteria are versatile heterotrophs associated with a variety of living and nonliving sources of organic matter. They have been isolated from seawater throughout the world--from tropical, temperate, and polar regions, and from surface waters to depths of several thousand meters (Orndorff and Colwell, 1981; Ruby et al., 1980; Shilo and Yetinson, 1979). Luminous bacteria may exist as mutualistic symbionts in the light-emitting organs of fishes (Herring and Morin, 1978), as enteric bacteria in a variety of marine organisms (Hastings and Nealson, 1977; Liston, 1954), as parasites of crustaceans (Baross et al., 1978; Harvey, 1952), as saprophytes on decomposing macroscopic animal matter (Harvey, 1952; Hastings and Nealson, 1977), or as members of the general planktonic microbial population of seawater (Hastings and Mitchell, 1971; Ruby and Nealson, 1978; Yetinson and Shilo, 1979). The bioluminescent organisms, widely distributed in the marine environment (Orndorff and Colwell, 1981; Ruby et al., 1980), may provide biological sensors through analysis and development of the luminescence system. Highly sensitive detection and response systems of fish and other macroorganisms have been reported but remain to be studied in detail.

An interesting application of the luminescence phenomenon in marine microbial ecology is the use of DNA probes for studying the ecology, physiology, and genetics of marine bacteria. The lux A and lux B genes of the luminous bacterium Vibrio harveyi, which code for the two subunits of luciferase, have been cloned and used as a probe for luciferase genes in other organisms (Potrikus et al., 1984). The probe was synthesized by nick translation with sulfur 35-labelled deoxyadenosine triphosphate (dATP), whose longer half-life provides more time for shipboard studies. Although sensitivity is not lost through the use of this procedure, exposure must extend for 2 or 3 days, rather than for a few hours, to obtain autoradiographs. Probes, such as the lux gene probes, can be used very effectively to identify and quantitate virtually any gene in situ.

The potential of the oceans for providing organisms of unusual and fascinating structure and function, e.g., sensory physiology, electrophysiology, and osmoregulation, is great, and the committee did not attempt to review every possibility in exhaustive detail. Those research areas discussed above offer only some of the potentially productive avenues of research for marine biotechnology.

Chapter 8

RECOMMENDATIONS

The committee found no evidence indicating that specific areas of research should be funded to the exclusion of others. Although opportunities to support important research are evident, obvious signs of great neglect were not detected in any field. Nevertheless, the committee concluded that a great deal more research in certain areas of marine biotechnology would be especially rewarding.

Although basic research that might be most valuable to ONR has been identified, scientific discoveries cannot be predicted. Thus, the committee's recommendations should not be viewed as proscriptions of any research.

The committee's recommendations fall under three headings: general categories, specific research areas, and techniques.

GENERAL

The committee believes that the development of marine biotechnology is dependent on advances in basic research. It therefore encourages ONR to adhere to its current philosophy of supporting the best research projects and to encourage innovative, basic research by single investigators or small groups, independent of perceived applications. Furthermore, ONR should publicize its program more extensively to enlarge its pool of applicants.

ONR should not neglect multidisciplinary or long-term research projects. The value of and need for interdisciplinary research for long-term development was recognized by participants in both conferences held by the committee, as well as by the committee itself during its deliberations. Thus, in addition to funding projects proposed by individual scientists, ONR should consider allocating a portion of its resources to projects involving teams of principal investigators representing diverse disciplines. Perhaps the best way to achieve this is to find a scientist with the needed breadth of knowledge and interest and to fund that person sufficiently to form such a group. University-industry cooperation should also be encouraged.

The committee also recommends that ONR support efforts to improve instrumentation and to refine specific techniques. These activities may be necessary for the advancement of knowledge in the basic research areas of interest.

The Navy has the mandate to develop and protect the oceans and marine environment. Thus, basic research that will advance marine biotechnology merits the attention of ONR and provides justification for ONR to assume responsibility for basic research in marine biology.

The committee therefore recommends that ONR consider possible applications to the marine environment when reviewing proposals for basic research. If ONR builds bases of expertise in diverse fields, investigators associated with ONR-supported research can provide advice on specific topics relevant to naval needs. To this end, it is appropriate for ONR to support research in many fields, such as genetics, immunology, cell biology, microbiology, biochemistry, biophyrics, and chemistry, where applicable to marine biology.

There is a need to maintain reference cultures of germplasm for comparative studies. Marine biotechnology will require a ready source of such material for research and development. Therefore, the committee recommends that ONR support the maintenance of marine microorganisms in depositories and collections, of which the American Type Culture Collection is just one example.

RESEARCH AREAS

The various areas of biotechnology are expanding almost explosively. Marine biotechnology, among the newest and most innovative, is also one of the least developed. The Navy has a direct and vital interest in this discipline and would benefit from knowledge of the components of life in the sea--the nucleic acids, proteins, and polysaccharides. More importantly, there is a need to learn the structure, function, physiology, metabolism, and systematics of whole organisms functioning in the ocean, especially in the deepest parts of the oceans and at the newly discovered hydrothermal vent areas. For modern biology to be applied to marine systems, these lacunae in knowledge must be filled. Thus, the greatest research need concerns basic principles upon which marine biotechnology can be founded.

Nucleic Acids

Research concerned with nucleic acids is of central importance to biotechnology and has potential for producing results that can be applied in many ways. Over the past 5 years, there has been a staggering increase in developments that, for the most part, were neither planned nor foreseen. More importantly, the origins of these findings can be traced to advances in fundamental knowledge, as well as to the development of new techniques.

The committee recommends that special consideration be given to two areas: (1) the structural chemistry and conformation of nucleic acids and (2) molecular genetics and techniques of genetic engineering.

(1) Knowledge of the structure and conformation of nucleic acids includes topics such as coiling and supercoiling, hybridization and probe techniques, and nucleoproteins and complexing of nucleic acids with proteins.

(2) The potential of genetic engineering for biotechnology is widely appreciated, and support for research concerned with its development is well justified. Cloning of specific genes can lead to the directed biosynthesis of biomaterials; genes can then probably be modified to alter proteins to suit specific applications. Basic research in molecular genetics ranging from regulation of gene expression to mutagenesis would help to achieve this goal.

Proteins

Protein molecules play a vital role in most of the many chemical reactions that take place in a living cell. For example, practically all enzymes are protein molecules, whereas their substrates can be sugars, nucleic acids, proteins, or simpler biomolecules. To understand life at a molecular level, it is necessary to understand the interactions between biomolecules, and protein plays the central role.

The committee recommends that special consideration be given to three areas: (1) protein structure and function, including membrane receptors; (2) immunological detection; and (3) exploration of unusual enzymes.

(1) The major focus in protein research is the relationship between protein structure and function. The structure is no longer regarded as a static configuration. Rather, the internal motion of the molecule is now recognized as very important. This subject is being studied from many angles, both theoretical and experimental, but progress is slow. Because of the importance of this relationship and its relevance to biotechnology, the committee recommends that ONR fund studies designed to elucidate it.

It is the very nature of receptor proteins in membranes to act as biosensors. They transduce a signal and transmit it across the membrane. As yet they have had no practical impact, but elucidation of new principles could be very important for any specific sensor.

(2) Antibodies can be generated to react immunologically with virtually any chemical. This is probably the only completely general technique for detecting molecules. In addition, the technique offers some latitude with respect to specificity. For example, monoclonal

antibodies may be highly specific, whereas polyclonal antibodies possess some diversity with regard to the chemicals recognized. Research in immunology, specifically on monoclonal antibodies and the antigen-antibody reaction, merits consideration for support by ONR. However, the committee recommends that ONR be carefully selective in its support of research in this field to avoid duplication of effort already undertaken or planned elsewhere. Immunological research of significance to the marine environment and marine life systems should be covered by ONR because of potential benefits to the Navy.

(3) Enzymes are present in organisms that occupy habitats characterized by extreme temperature, pressure, or other properties. Some of these enzymes are likely to differ from those found in more normal habitats as a result of evolutionary adaptations (Zaborsky, 1982). The exploration of new and unusual enzymes (e.g., thermophilic enzymes and oxygenases) should be encouraged. Although the sensitivity of enzymatically based biosensors may be surpassed by immunological methods, specific applications and unusual enzymes may warrant additional research.

New methods for modifying enzymes and proteins need to be developed. These should include techniques based on organic chemistry and on the use of enzymes. The activity of enzymes in nonaqueous solvents should be explored, as should the characterization and use of immobilized enzymes.

Polysaccharides

Because of the structural diversity of the polysaccharides, which may provide a means of cellular recognition, these polymers may be very important in the development of biosensors and biomaterials. It also seems likely that important biomaterials could be generated by altering them. Recent technological advances have opened the door to understanding and modifying the structure of polysaccharides, and ONR has an opportunity to become a lead agency in the field of marine polysaccharide research.

The committee recommends that special consideration be given to three areas: (1) understanding the structure of polysaccharides; (2) understanding and cataloging their specificities; (3) and searching for new and unique polysaccharides.

(1) Polysaccharides have more complex structures than proteins and nucleic acids. It is necessary to understand both the fine structure of the polymers themselves and the ways the structures are linked together to form the polymers. Various new technologies, such as nuclear magnetic resonance and circular dichroism, will be useful in such studies. In addition, studies of the three-dimensional structure of polysaccharides will be very important for understanding their potential applications.

(2) Aside from the major bulk materials cellulose and starch, and a number of widely distributed marine polysaccharides, major biological specificity, especially of cell surfaces, resides in the multitude of small polysaccharides. This specificity should be valuable in the construction of specific sensors.

(3) There is a great variety of naturally occurring polysaccharides, and many of them have proved useful (see Table 6-1). A search for new and unique polysaccharides should be initiated in all areas of the biological world, especially the oceans.

Synthetic Analogs

Research on synthetic analogs and systems encompasses the major disciplines of chemistry, biophysics, biochemistry, and molecular biology. The term synthetic analog refers to the specific design of discrete compounds that mimic a particular function and structure of a biological molecule. The term synthetic system refers to an ordered array, i.e., a molecular arrangement of different compounds integrated into a functioning unit. A good example of a synthetic system is a synthetic membrane comprising protein and lipids. Although research on synthetic analogs and systems has been conducted with some success, recent advances in molecular biology, organic synthesis, materials sciences, instrumentation, and computational capabilities open up new vistas. For example, there are opportunities for synthesizing, characterizing, and improving analogs of enzymes, membranes, photocatalysts, electron transfer agents, ion-transport proteins, chelating agents, hormones, and many other compounds of biological importance.

The committee recommends that special consideration be given to two areas: (1) specificity of the biological activity of synthetic analogs and systems and (2) their activity in unusual microenvironments, including nonaqueous solvents.

(1) Studies are needed to improve the design and synthesis of biomimetic molecules exhibiting very highly specific interactions with the natural ligand molecules. Synthetic analogs may be operative in microenvironments different from those in which the natural molecules operate and may exhibit chemical stability superior to that of the natural molecules in those microenvironments.

(2) The synthesis and characterization of analogs and systems exhibiting other than hydrolytic phenomena should be supported. To date, most studies have focused on hydrolytic reactions of esters and amides.

Whole organisms

There are many important research areas in marine biology, especially marine microbiology, that involve studies of intact cells

and whole organisms. An understanding of the diversity of the physiology, structure, ecology, sensing and communication ability, and systematics of marine organisms will enhance understanding of cellular and biochemical processes. Moreover, such work will probably provide direct insights that could be applied to the development of biosensors and biomaterials.

The committee recommends that special consideration be given to three areas: (1) structure, function, and metabolism of unusual organisms, especially those found in extreme environments; (2) sensory detection and communication, e.g., electromagnetic detection in fishes; and (3) systematics, physiology, and ecology of marine microorganisms.

(1) The ONR should fund basic research on the structure, function, and metabolism of archaeobacteria, hydrothermal vent organisms, and other species of marine animals, plants, and microorganisms not well known or recently discovered. These include bioluminescent, oligotactic, magnetotactic, psychrophilic, barophilic, oligotrophic, and related species. High temperature enzymes, bioluminescence systems, and the enzymatic mechanisms involved in chemosensing may be a valuable source of new findings for biosensor and biomaterial development.

(2) Electromagnetic detection in fishes is an intriguing area of research. Studies on this subject presently involve an interdisciplinary interface between biophysics and biology. The committee recommends support for this research because of its great potential, especially if it proves to be both valid and feasible for development.

(3) The committee also recommends research on the systematics of marine microorganisms, especially if it is molecularly based, to provide a data base concerning their characteristics and distribution. This would also provide a basis for studying ecological patterns of marine microorganisms, which the committee believes to be important. These patterns include community structure, trophic relationships, and energy flow. Studies of this type would be helpful in determining the source of foreign objects and in biosensing.

TECHNIQUES

Techniques are tools needed in basic research. Major advances in any field are dependent on them. For example, the understanding of protein structure depended upon x-ray crystallography, gel electrophoresis, and radioimmunoassays. Projects need not be limited to the development of methods and techniques, but should include significant basic research as well. Indeed, the line between the development of techniques and basic research is not a clear one.

The recommendations that follow all arise from considerations of the five research areas described above. They share an emphasis on techniques--methods and instrumentation--and are all motivated by fundamental research questions.

The committee recommends that special consideration be given to six areas:

(1) transducers such as metal oxide semiconductor field-effect transistors (MOSFET) to convert the antigen-antibody reaction into an electric signal in real time;

(2) use of DNA probes, which can lead to new insights into marine systems, e.g., by improving sensitive detection in the noisy marine environment;

(3) amplification of a signal by coupled and consecutive reactions in biocatalytic and physical cascades;

(4) the development of new instrumentation, e.g., spectroscopic analyses for synthetic analogs and mechanisms for determining the structure of molecules such as polysaccharides, leading to a better understanding of many developmental problems related to cell recognition and to the development of biosensors based on the same principles;

(5) new and specific methods of fragmenting native polysaccharides at known points, using rational methods similar to those used in the design of catalysts that can cleave a linear DNA molecule at a specific point, and new physical methods for examining both the intact polysaccharide and the fragments; and

(6) manipulation of organisms from extreme or special environments.

Apparatuses have been developed to maintain high hydrostatic pressure, salinity, temperature, and other important environmental parameters, but greater refinement is needed for retrieval, maintenance, and observation of and experimentation on these organisms. Of particular interest are organisms that are symbiotic, phototropic, luminescent, halophilic, psychrophilic, thermophilic, acidophilic, barophilic, microaerophilic, or anaerobic. Research involving the development of methods for isolation, culture, and maintenance of such organisms is strongly encouraged.

In summary, ONR should invest selectively in basic research, especially when it involves marine material, concerns the marine milieu, and shows promise of leading to the development of new methods and applications in biotechnology. The marine environment is indeed the province of the Navy, and a mastery of modern biochemistry and molecular biology is necessary to understand the marine realm, as is the knowledge of the structure and function of organisms that live at

high pressure or temperature. The committee has provided a focus for future research, fully realizing that the Navy cannot support all aspects of marine biotechnology but that it can promote the development of a field ripe for exploitation. By supporting the best scientists and the most innovative research projects, ONR will reap the best rewards for its funding.

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