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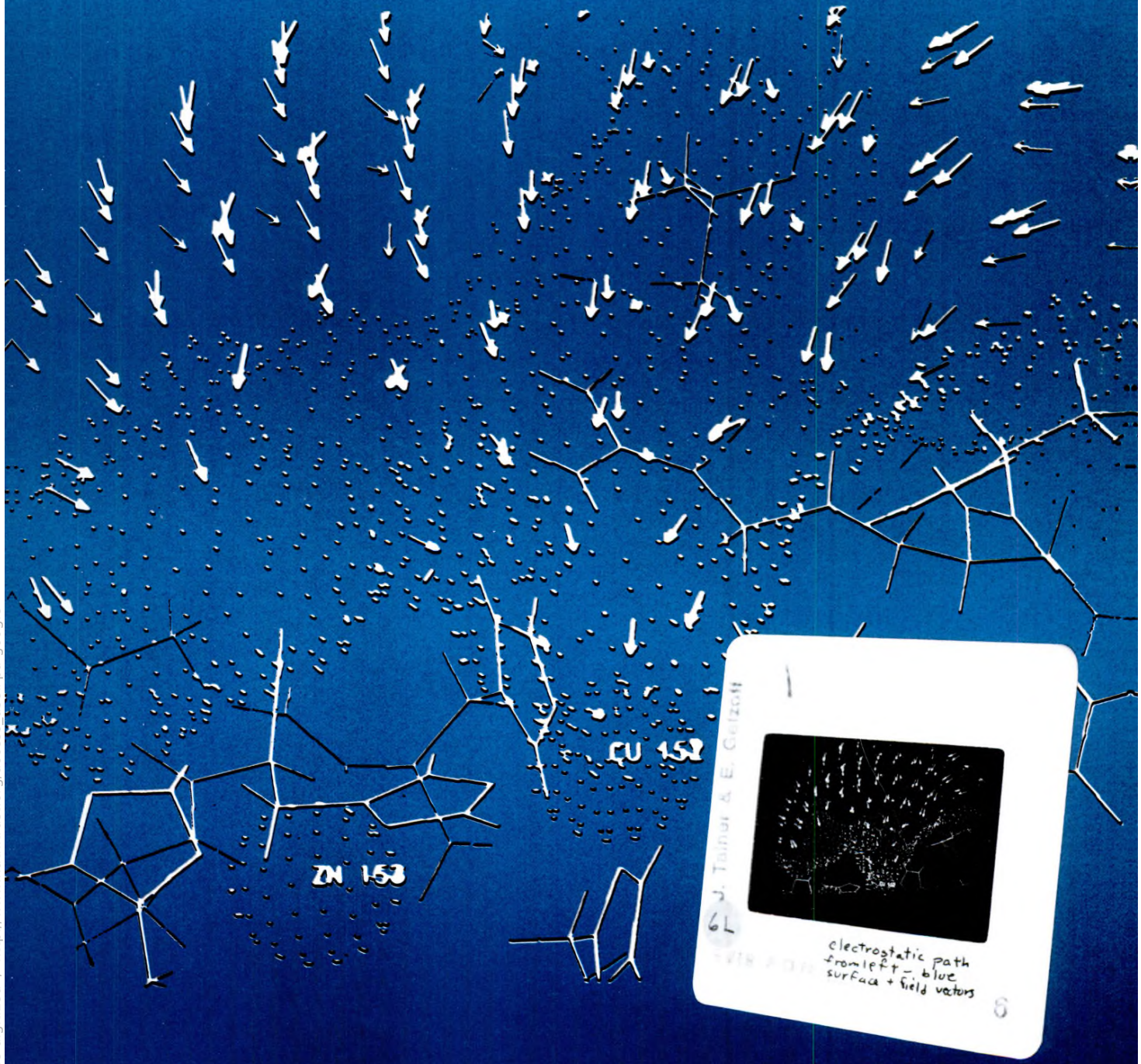

Zoothamnium (magnified bacteria growth) on aluminum after 15 days. Professor Ralph Mitchell of Harvard University with funding from the Office of Naval Research (ONR) is studying the role of bacteria in the marine corrosion process and hydrogen embrittlement of metal. His research is providing for the first time a clear understanding of specific corrosion reactions that are mediated biochemically by microorganisms. The ultimate goal of the research is to gain the ability to control biologically induced corrosion.

Specifically Professor Mitchell's research is focusing on hydrogen sulfide production, hydrogen embrittlement, organic acid production, iron and maganese oxidation, and sulfuric acid production.

The corrosion of metals in contact with seawater is a major source of operational problems and economic loss to the Navy. In the United States, microbial corrosion of equipment amounts to an estimated yearly loss of seven billion dollars. ONR has been supporting research for many years in this field, and now the results are encouraging for improving corrosion related problems in the future.

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Articles

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About the Cover

Arrows showing electrostatic field guiding substrate to the reactive copper in the active site channel of Cu, Zn superoxide dismutase. This computer representation uses state-of-the-art technology to visualize and understand the physical laws and properties that govern enzyme reactions. Such information will yield an understanding of enzymatic catalysis and will ultimately enable us to control or alter enzymes for useful purposes. This photograph was produced by Elizabeth Getzoff, John Tainer, and Michael Connolly of Scripps Clinic; Robert Langridge, Paul Weiner, and Peter Killman of the University of California at San Francisco; and David and Jane Richardson of Duke University. (See article beginning on page 3.)

Naval Research Reviews publishes articles about research conducted by the laboratories and contractors of the Office of Naval Research and describes important naval experimental activities. Manuscripts submitted for publication, correspondence concerning prospective articles, and changes of address, should be directed to Code 732, Office of Documents, U.S. Government Printing Office, Washington, D.C. 20402. *Naval Research Reviews* is published from appropriated funds by authority of the Office of Naval Research in accordance with Navy Publications and Printing Regulation NAVSO P-35.

Introductory Note

In the last four years, there have been four issues of *Science* devoted to the "revolution" in biology. The two most recent were entitled, "Biological Frontiers," (*Science*, vol. 222, 1983, pp. 657-860) and "Biotechnology" (*Science*, vol. 219, 1983, pp. 609-747). The Biological Sciences Division of the Office of Naval Research (ONR) plays a significant role in this "revolution" through its support of basic biological research.

A logical question is, what is the reason for this surge in biology? I think a quotation from the introduction by Dr. F. Blattner to "Biological Frontiers" in *Science* is appropriate. Referring to the biological revolution which is really an explosive advance or an evolution rather than a revolution, he states: "It has not been necessary, as was the case with quantum theory or relativity, to overturn major paradigms of accepted thought; rather, an enormous increase in the power of experimental techniques is now producing answers to complex long-standing biological questions."

These techniques were contributed mostly from molecular biology and include gene cloning, recombinant DNA, DNA and protein sequencing, and monolonal antibodies. It is important to emphasize that the techniques were developed by molecular biologists, but it is the whole of the biological community that uses these to solve long standing problems of biology, such as cell movement, cellular communication, organ formation and organismal behavior.

The biological community is large and diverse. As a consequence the research areas covered are numerous. In this issue of *Naval Research Reviews*, we have selected only three areas for review. All are ones which the Biology Division at ONR are emphasizing.

Since its inception in 1946, the Office of Naval Research has had an active program supporting basic research in the biological sciences. The objective of the program is to provide the fundamental knowledge from which bioenvironmental and biomedical developments are derived. A great deal of diversity exists in the research supported. The division is organized into three groups, with the overlap in interest that is expected in biological research.

The *Molecular Biology Group* supports research where the goal is an understanding of biological phenomena at the molecular level as well as increasing our understanding of the biochemistry that forms the basis for biotechnology. The particular areas of emphasis over the next several years are in receptor channel pump matrices, stable immobilized enzymes, novel biopolymers, biocorrosion, biological molecules for materials application, and biological electron conducting systems.

The *Cellular Biosystems Group* supports research directed at understanding cellular responses to toxic and stressful environments. Studies range from membrane and cytoplasmic changes in trauma states to interactions of macromolecules, cells, and whole organisms with electromagnetic fields. Electromagnetic fields are also of interest in terms of their capacity to enhance tissue growth and provide information about biological processes.

In addition, this group is the focal point of studies on cellular immunology and immunopharmacology. The objective in these areas is to enhance the effectiveness of conventional vaccines, to find an alternative to the use of toxic antigens in vaccines, to enhance natural immunity as a substitute for vaccines, and to explore new directions in basic cellular immunology. A developing program is investigating neuronal regulation of immunity.

The *Physiology/Neurobiology Group* supports research directed at understanding fundamental physiological and behavioral process in both normal and adverse naval environments. Program interests are focused on learning and memory, information processing, physiological responses to operational stresses, and the neurophysiological/neurochemical control mechanisms involved in immunity or circadian periodicity. Additional program interest in biocybernetics and central nervous systems (CNS) injury are also addressed.

Robert W. Newburgh
Head Biolgical Sciences Division, ONR

Structure-Function Relationships in Supramolecular Biological Systems

by
Eli D. Schmell and Steven E. Kornguth
Office of Naval Research

The technical and conceptual advances achieved by basic scientists in the field of molecular biology during the past three decades have created a new area of basic, applied and industrial research, biotechnology.

Table 1

BIOTECHNOLOGIES

1. Genetic Engineering
Isolation of genes from one organism and insertion into a different organism allowing the inexpensive production of defined molecules by bacteria.

2. Protein Engineering
The synthesis of novel polypeptides or the chemical modification of existing proteins for the production of catalysts, adhesives and bioreceptors.

3. Cell Fusion
Fusion of two different cell types to produce a hybrid that exhibits new properties. The fusion of tumor cells with lymphocytes results in stable cell factories that produce monoclonal antibodies.

4. Immobilization of Biopolymers
Attachment of functional biopolymers or cells to water insoluble matrices. Immobilization often stabilizes the biopolymer and facilitates separation of catalysts from reactants.

The national interest in this emerging technological base is exemplified by recent reports from the Congressional Office of Technology Assessment,[1] two recent issues of *Science*[2] that were totally dedicated to modern molecular biology, and a review of research opportunities in marine biotechnology.[3]

Research questions that are being investigated by ONR contractors in this area of basic science include:

(a) the molecular basis of vision;
(b) the *de novo* synthesis of polypeptide catalysts and the correlation of their activity with the molecular structure of naturally occurring enzymes;
(c) the synthesis of efficient electron transfer materials based on porphyrin systems;
(d) the production of novel adhesive biopolymers by genetic engineering;
(e) the isolation of chemical or biological receptors and their reconstitution into defined synthetic membranes;
(f) the molecular basis for segregation of genetic information and control of expression in marine organisms;
(g) the molecular basis of cellular resistance to unusual environments such as high temperature, salt, and pressure.

Genetic engineering, protein engineering, cell fusion technology and biopolymer immobilization provide the technologies necessary to extend our knowledge of structure-function relationships in biological systems. These technologies are summarized in Table 1.

Membrane Associated Enzymes and Receptors

One primary goal in molecular biology is the correlation of the function of biopolymers with their molecular structure. Many biopolymers that function as catalysts, ligand binders, ion channels and electron transfer materials are associated with lipid bilayer membranes. The study of structure-function relationships in such membrane systems therefore requires an analysis of surface chemistry and molecular interactions that permit catalytic function. The macromolecular unit involved in a chemical reaction, for example an enzyme catalyzing an oxidation-reduction reaction, is often composed of three components: (1) a low molecular weight moiety such as a porphyrin that transfers electrons; (2) a large molecular weight structure such as a protein that determines which specific chemical substrates will donate or accept the electrons; and (3) the supramolecular structure of the membrane-protein complex, which provides the appropriate dielectric medium and surface organization necessary for the function to be realized (Figure 1). Retinal, an isoprene derivative essential for vision, is

Figure 1

Chemical components of electron transfer porphyrin systems. Several cytochromes from animal and plant cells exist in membrane associated forms.

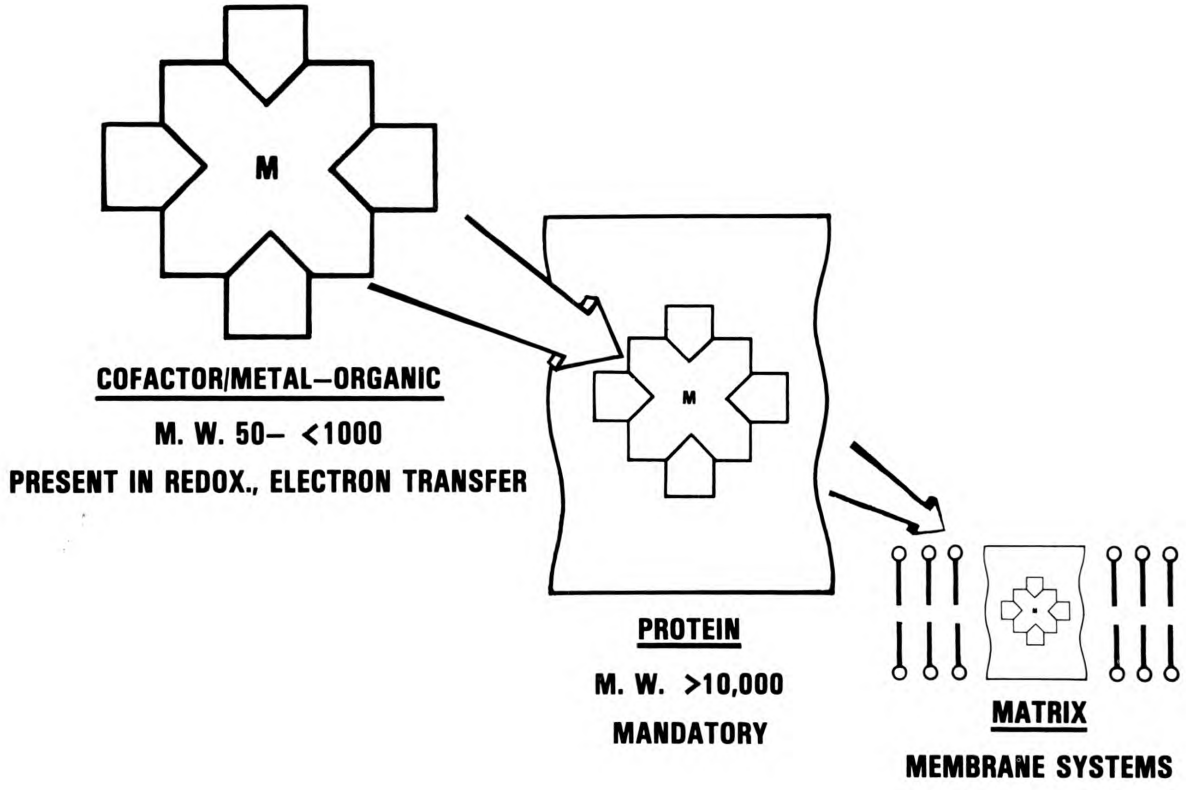
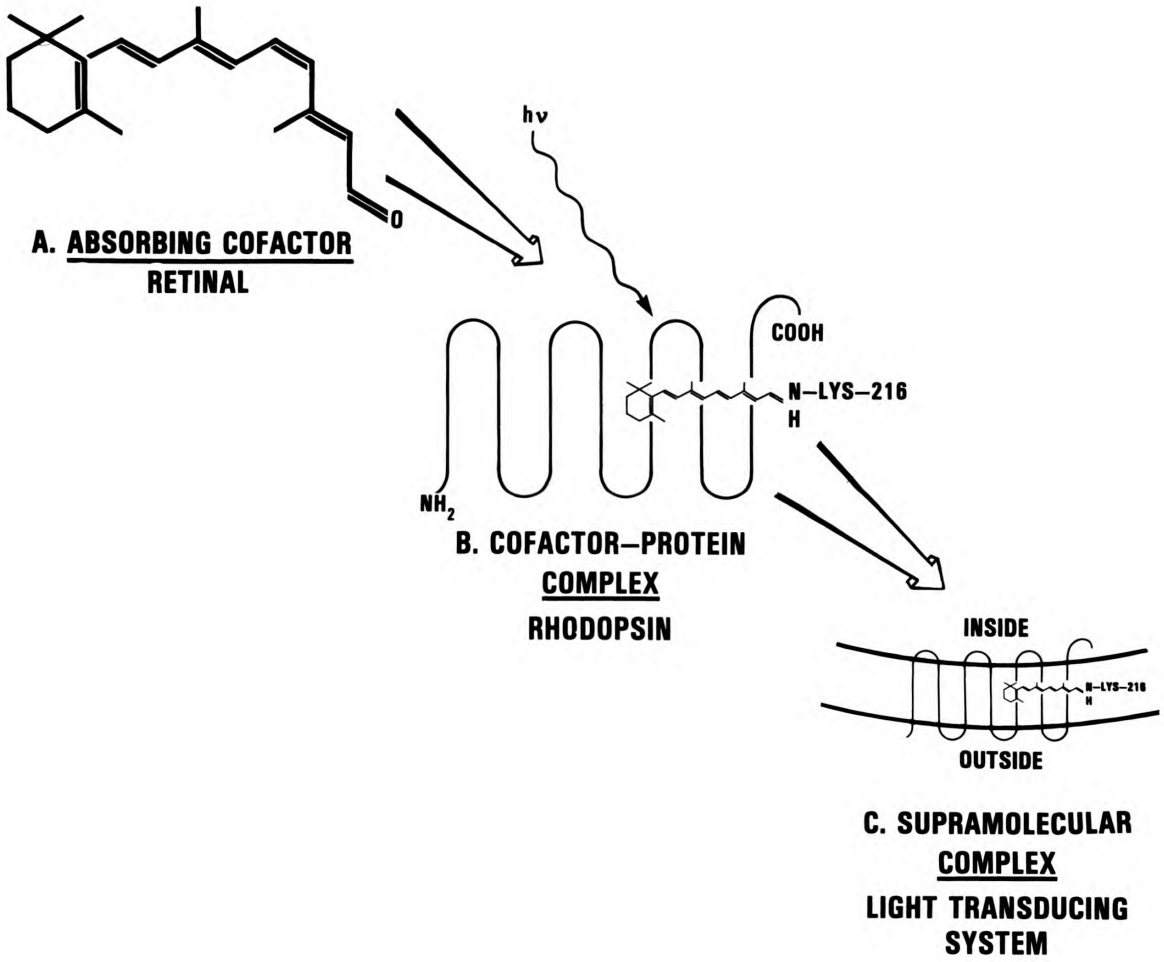


Figure 2

Supramolecular complex for biological light transduction. In living organisms light is absorbed and transduced by a highly organized complex of molecules. The absorbing chromophore is the low molecular weight cofactor, retinal (A). The wavelength of light monitored by a particular system is modulated by the microenvironment of retinal which is a function of the amino acid composition of the rhodopsin protein (B). The exact 3-D structure of the rhodopsin-retinal complex is maintained by its orientation in a cellular membrane (C).



another example of a low molecular weight constituent that is important in the function of a membrane associated protein system, opsin. Retinal is important in the detection of light by photoreceptor cells in the eye and in bacteria; it functions in conjunction with the protein opsin in the low dielectric medium of the membrane. The membrane matrix imposes the appropriate structural orientation on the opsin and provides a low dielectric medium (Figure 2).

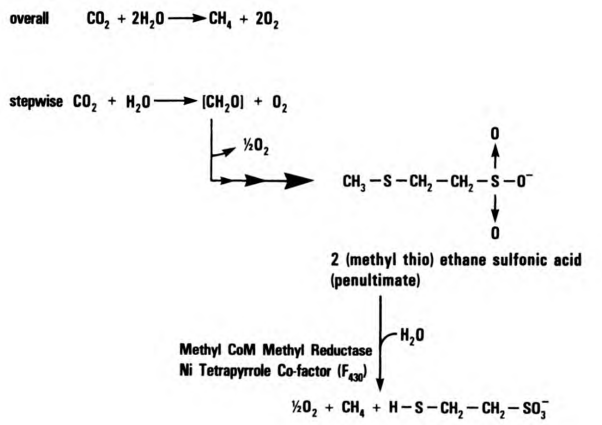
Extensive research on the porphyrin-containing enzymes reveals a general principle in biology. The principle is that a strategy, used successfully to solve a particular problem faced by biological organisms, is retained and modified to solve related problems. For example, the iron-porphyrins in hemoglobin bind oxygen and carbon dioxide in a reciprocal manner and are used to transport these gasses in the blood. The porphyrins are also involved in the reduction of 0_2 to 0^{-2} when associated with the enzyme cytochrome oxidase as a copper derivative. They participate in the reduction of carbon dioxide to methane in methanogenic bacteria as part of the enzyme system, methyl Co M methyl reductase[4-6]. In this last system, nickel is the metal constituent. In each of the above examples, the porphyrin group is the ligand binder and site for electron transfer. The protein that is associated with the porphyrins determines the specific substrates that donate and accept the electrons. Thus, the function of the system is governed by each of its structural components (Figure 1).

Scientists from Duke University (Dr. A. Crumbliss), North Carolina State (Dr. R. Bereman) and University of North Carolina (Dr. M. Crenshaw), are investigating the mechanisms by which *Methanobacterium thermoautotrophicum* reduce carbon dioxide to methane (Figure 3). The penultimate step requires a nickel porphyrin, F_{430}, and a protein catalyst for activity. This reaction is of interest for several reasons. It involves: (1) a paramagnetic enzyme; (2) an unusual example of a nickel porphyrin catalyzed reaction; (3) an enzyme system exhibiting strong light absorbance; and (4) the transfer of hydrogen from water to a methyl moiety. This system is a good model to explore various questions related to the biomaterial sciences.

To understand structure-function relationships, the Molecular Biology Program at ONR suports research that examines three aspects of molecular structure: (1) the interaction of functional groups with substrates or with environmental signals and the molecular basis of that interaction; (2) the role of the biopolymer structure in performing the function (e.g., relation of opsin structure to light absorbance at a particular wavelength); (3) the chemical requirements of the matrix which facilitates biological function in supramolecular systems. The structure-function relationships of proteins are investigated by protein engineering, genetic engineering and immobilization strategies.

Figure 3

Anaerobic reduction of carbon dioxide to methane by methanogenic bacteria. The penultimate reaction is catalyzed by the F_{430} porphyrin containing enzyme methyl Co M methyl reductase.



Three strategies are used to approach the structure-function relationships in methyl Co M methyl reductase. One approach involves the sequential substitution of other metals in place of nickel; the second utilizes modification of the porphyrin moiety; and the third is the immobilization of the metallo-porphyrin complexes on electrodes in the absence of protein. In each of the above strategies, one element of the electron transfer complex is altered and its effect on electron transfer is determined. Other studies have shown that the metalloporphyrins are important organic conductors. The efficiency of electron transfer in these systems is a function of the distance between and orientation of the porphyrin moieties. James Collman et al[7] from Stanford University have shown that face to face porphyrins, spaced at 6° distance, are efficient electron transfer agents. Larger or smaller distances between the porphyrins reduce the efficiency of this process. Makinen and colleagues[8] recently demonstrated that the kinetically slow steps of electron transfer appear to be regulated by the perpendicular placement of porphyrins, as in the cytochrome cd_1 of *Pseudomonas aeruginosa.* These two observations provide a biological model for modulating electron conduction in porphyrin containing polymers. A related class of organic electron conducting polymers, the phthalocyanines, was reviewed in an earlier volume of this series.[9]

As with the porphyrin system described above, the visual pigments used to detect light and color are also complexes of low molecular weight components associated with proteins and membranes. The chromophore molecule that absorbs light in bacteria and in mammalian rod cells is retinal. As a consequence of light absorption, the retinal isomerizes from a cis configuration at carbon 11 (Figure 2) to a trans configuration. Although retinal in the rods absorbs the light, the specific wavelength monitored is determined by the interaction of the retinal with the protein opsin. The maximal wavelength response to red or green or blue by the photoreceptor of the rod cells is apparently determined by small modifications of opsin structure. The specificity of the light-sensing reaction is therefore a function of polymer structure. The supportive membrane in which the rhodopsin resides influences whether the light detecting reaction will occur. The retinal protein complex must be in a matrix of low dielectric constant (i.e., a lipid membrane) for light absorption to cause the regenerative cis to trans isomerization of retinal. In this light absorbing system, the membrane matrix serves to assure the appropriate three-dimensional configuration of the rhodopsin and provides the appropriate low dielectric medium.

Protein Engineering

One long-range goal of the protein engineering effort of the Molecular Biology Program is the design and synthesis of novel and altered polypeptides in order to provide efficient catalysts and specific binding proteins which are not readily available in nature. Two complimentary approaches are being pursued to realize this goal.

The first involves genetic engineering approaches to the generation of mutant or altered protein molecules using site-specific mutagenesis. This methodology is being applied by Dr. G. H. Khorana and co-workers at the Massachusetts Institute of Technology to the membrane associated light absorbing proteins (opsins) from bacterial and mammalian systems. Dr. J. Kraut and co-workers, at the Agouron Institute in San Diego, are using the same technique to study the metabolic enzyme dihydrofolate reductase (DHFR). In a third program, Dr. J. Richards and co-workers at the California Institute of Technology are studying the penicillin degrading enzyme, B-lactamase.

A second approach to the engineering or design of proteins is the preparation of novel polypeptides using solid phase organic synthesis. Drs. B. Erickson at the Rockefeller University and J. Richardson at Duke University are synthesizing model catalysts and analyzing their structural properties by x-ray diffraction. Both of these approaches to protein engineering are described in somewhat greater detail below.

Site-Specific Mutagenesis

Genetic engineering technology currently enables molecular biologists to manipulate the structure and expression of genes. The proteins which are the products of such manipulated genes can be altered to an unprecedented degree. In particular, site-specific mutagenesis allows the alteration of individual amino acid residues in polypeptide chains.[10] This method is evolving rapidly as a powerful tool for the elucidation of structure-function relationships in proteins and enzymes. The technique involves selective replacement of one base in a gene with another. Expression of such an altered gene results in a protein with a single, specific, amino acid replacement (Figure 4). Described below are two experimental efforts that are supported by the Molecular Biology Program of the Office of Naval Research, to elucidate protein structure-function relationships by genetic engineering.

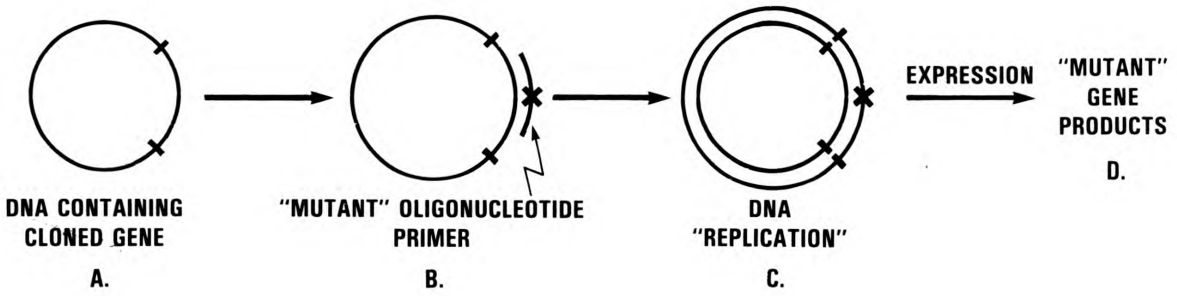
Rhodopsin and Bacteriorhodopsin. Opsins are light transducing proteins currently under investigation in Dr. G. H. Khorana's laboratory at the Massachusetts Institute of Technology. The chemistry of these proteins has recently been reviewed.[11] Rhodopsin, a 39,000 dalton protein, is the main visual pigment in rod outer segments of the retina in both vertebrates and invertebrates. Similarly bacteriorhodopsin, a 27,000 dalton protein, catalyzes light-driven proton translocation across the purple membrane in several halophilic bacteria. The resulting electrochemical gradient can be exploited by the cell for useful work. In both systems, retinal is the light absorbing pigment. The pigment is linked to the protein opsin via a lysine residue. It is the isomerization of retinal from the cis to the trans configuration which is the initial step in photon capture and light transduction (Figure 2).

Although retinal is apparently the universal light-absorbing pigment in essentially all visual systems, absorption maxima vary from 350nm to 620nm. Clearly, the wavelength of light absorbed by the pigment is affected by the chemistry of the microenvironment it experiences. This is dependent on the structure of the opsin molecule to which the retinal is attached. Thus, the light absorbing system is an excellent model for studying structure-function relationships in polypeptides.

Recently, Dr. Khorana's group has purified and sequenced the bacteriorhodopsin molecule and developed an *in vitro* reconstitution system to study the function of the purified protein at the molecular level. The gene coding for bacteriorhodopsin has also been cloned and expressed. Thus, the bacteriorhodopsin molecule can now be manipulated structurally using genetic engineering. The effects of these alterations on the protein's function can be observed using the *in vitro* reconstitution system. Using a photosensitive retinal analog, the

Figure 4

Schematic representation of site-specific mutagenesis. A particular modification can be engineered into a desired, cloned gene (A) by synthesizing a complimentary oligonuclotide primer with a desired nucleotide replacement (indicated by an X in panel B). The oligonucleotide primer is extended by DNA synthetic enzymes to yield a complimentary DNA molecule containing the altered or mutant gene (C). Following insertion and expression in a bacterial vector, the altered gene product is obtained (D).



amino acids in rhodopsin that are oriented in close proximity to the retinal cofactor have been identified.[12] Dr. Khorana's group is now generating bacteriorhodopsin molecules with alterations of these amino acids to determine the structural requisites associated with the light transducing function of the molecule.

Dihydrofolate Reductase. Dr. J. Kraut and his coworkers at the Agouron Institute and the University of California at San Diego have been studying the molecule, dihydrofolate reductase (DHFR). DHFR is a soluble 16,000 dalton enzyme which catalyzes the NADPH dependent reduction of dihydrofolate to tetrahydrofolate. The investigators have isolated DHFR from several prokaryotic and eukaryotic sources. They obtained high resolution, three-dimensional structural maps of DHFR by x-ray crystallographic techniques.[13] Recently they have applied the site-specific mutagenesis approach to addressing basic questions concerning the amino acid sequence, the three-dimensional structure, and enzymatic activity of DHFR. In particular, they have produced three specific, mutant DHFR molecules to determine the significance of particular amino acid residues in the enzyme's functional properties.[14]

The three mutations produced were based on specific information concerning the enzyme's active site composition, the catalytic mechanism, as well as the three-dimensional structure of the molecule (Figure 5).[13,14] The carboxyl moiety of aspartic acid number 27 (ASP 27) is apparently involved in stabilizing a carbonium ion intermediate that is formed during catalyses. The investigators altered this amino acid to an asparagine residue (ASN 27). This modification removes the anionic character of the carboxyl moiety of residue 27 with minimum change in the size of the molecule. This mutant molecule was found to be essentially inactive, implying that a charged carboxyl group is essential for activity. Similarly, a glycine residue was replaced with an alanine residue at position 95 (ALA 95). The glycyl-glycine dipeptide, at positions 95–96, is considered essential for normal enzyme activity since it apparently orients the carbonyl function of Ile 94 which bonds to the cofactor, NADPH.[13] This ALA 95 mutant was completely inactive, suggesting the glycyl-glycine portion of this tripeptide is indeed essential. Finally, proline residue 39 was modified to a cysteine (CYS 39). This mutation was chosen since the three-dimensional structure revealed that position 39 is opposite a cysteine residue in position 85 (CYS 85). The distance between positions 39 and 85 is appropriate for the formation of a disulfide bond between two cysteines. Analysis of the CYS 39 mutant revealed that inter- and intra-molecular disulfide bonds are formed, and the oxidized form of the enzyme is approximately 50 percent as active as the natural enzyme (i.e., with a proline at position 39). Thus, the constraints placed on the molecule by forming the disulfide bond between CYS 39 and CYS 85 reduce the enzyme's activity.

The results of these studies and future experiments utilizing this technique, as well as related genetic engineering methodologies, should yield important insights into structure-function correlation in protein molecules.

Figure 5

Schematic representation of dihydrofolate reductase and of enzyme-substrate bonding. The backbone structure of DHFR from E. coli is shown on the left, with the approximate location of every tenth amino acid residue indicated. The circles show the position of the amino acids which were altered by site-specific mutagenesis. To the right is an expanded view of the dihydrofolate substrate interaction with the active site isoleucine 94 and aspartic acid 27. The arrows indicate the sites which are reduced by enzyme catalysis. The drawing is adapted from the work of Villafranca et al[14] and the hydrogen bonding is extrapolated from the data of Bolin et al.[13]

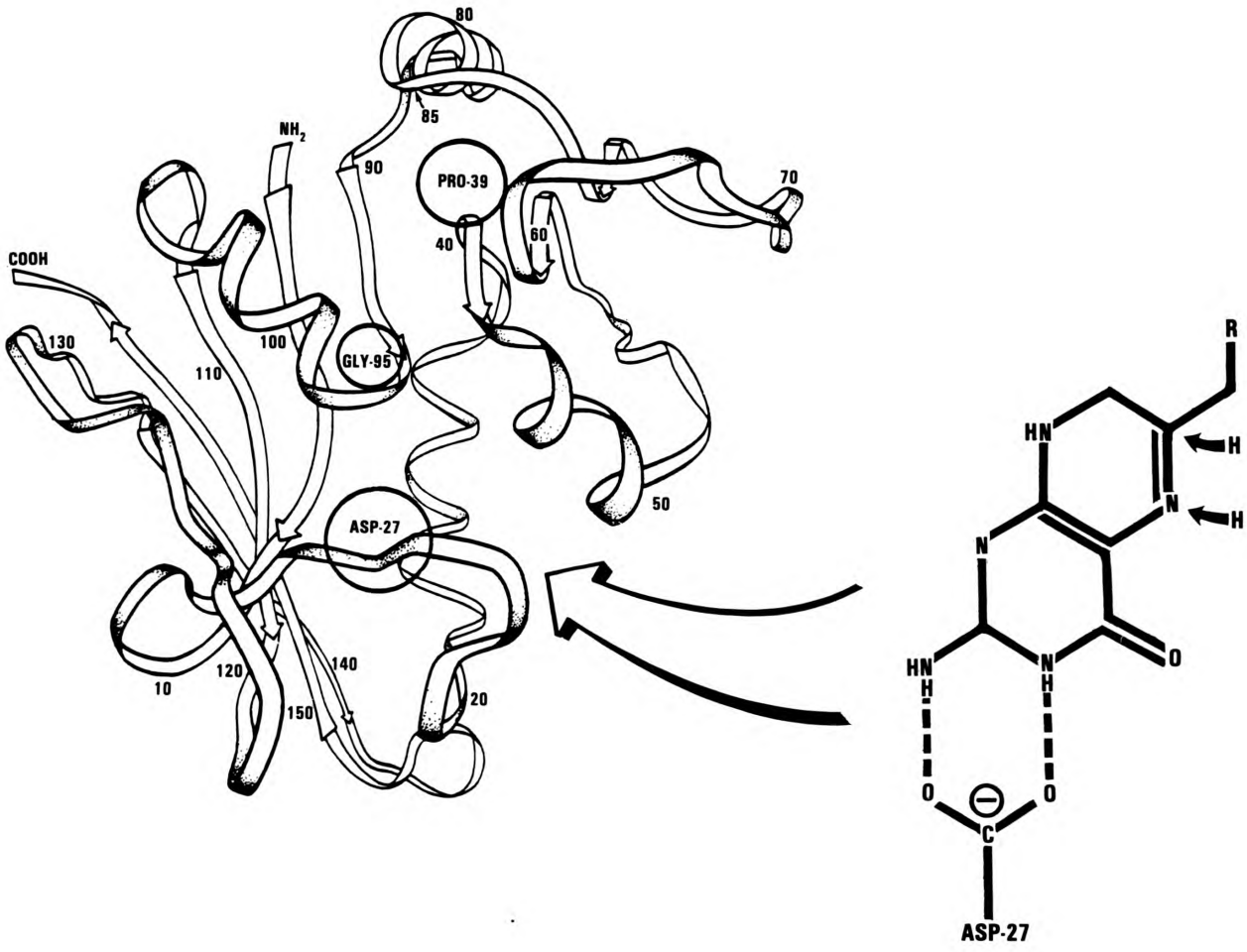
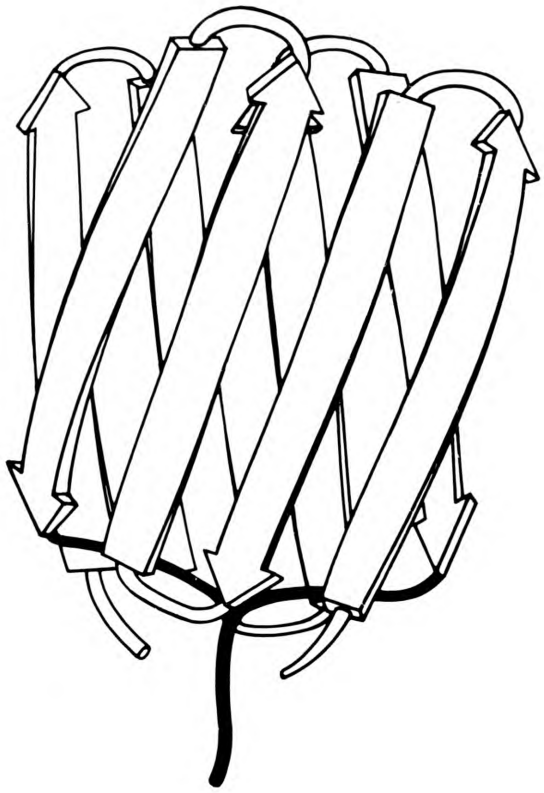
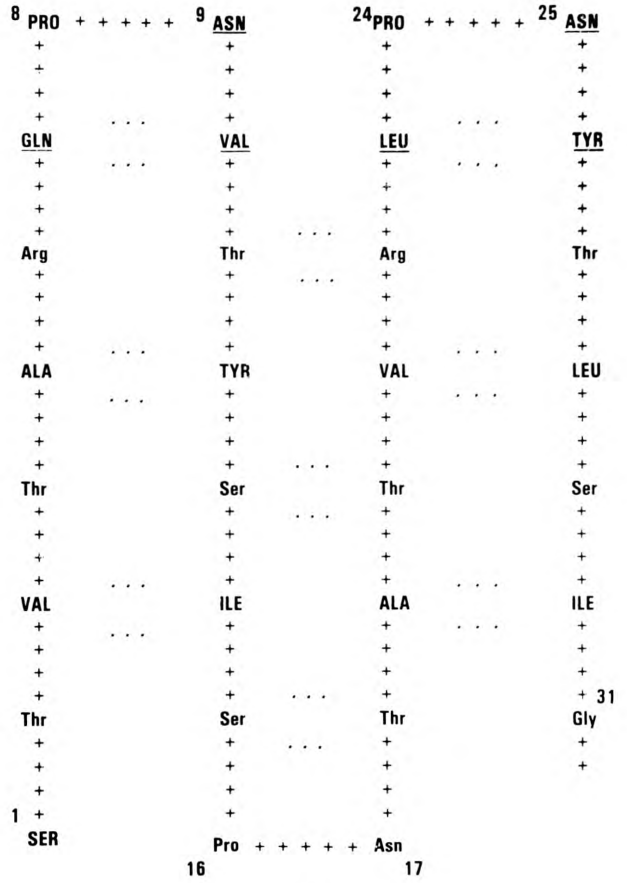


Figure 6

Betabellin. A schematic representation of the predicted three dimensional structure of betabellin is presented in (A). The structure is composed of a dimer joined covalently at the bottom by chemical crosslinking. The monomer is composed of four anti-parallel β-pleated sheets. The amino acid sequence of the monomeric unit is shown in (B).



A



B

De Novo Synthesis of Novel Polypeptides

A second approach to the study of enzyme structure, the *de novo* synthesis of novel polypeptides, is a collaborative effort between two investigators, Drs. B. Erickson of Rockefeller University and J. Richardson of Duke University. The investigators have designed and synthesized a novel protein having an amino acid sequence which they propose should fold into a particular three dimensional structure.[15] This novel protein will be engineered to contain a desired binding or catalytic site. Producing such a molecule involves the design of small peptides having predicted patterns of chain folding. Prediction of the folding patterns for such peptides utilizes computer graphics, which are based on a correlation of primary amino acid sequences, and corresponding x-ray crystallographic structures from naturally occurring proteins. The *de novo* protein engineering project is a direct approach to protein design and synthesis rather than a modification of the structure of existing proteins.

The first phase of the project involves the design of the protein. The structure of the molecule is based on a combination of methods for the prediction of structure including: (1) the construction of plastic space filling molecular models; (2) the heuristic prediction of secondary structure; and (3) modeling of side chain interactions using computer graphics. The design of the structure also includes consideration of the chemistry of the synthetic and sequencing strategies. These considerations lead the investigators to choose an initial structure known as a β-barrel. The particular structure to be synthesized is called betabellin and is shown schematically in Figure 6A. The structure was designed to have strong and regular hydrogen bonding within each strand. The structure will have internal two-fold repeats (i.e., a dimer) which would be most desirable for two reasons. First, this would simplify synthesis of the molecule. Second, symmetry within the structure will enhance the probability of obtaining crystals, an absolute requisite for subsequent x-ray analysis of the peptide's structure.

The molecule has been synthesized as a dimer. This was accomplished using a crosslinking agent specifically designed with two-fold symmetry in the region of peptide synthesis. The cross-linking agent will also facilitate the incorporation of a heavy atom such as iodine into a well ordered, defined position. The heavy atom permits the solution of the crystal structure of the molecule.

Eight amino acid residues per strand should provide sufficient primary sequence to obtain a stable structure that contains a catalytic site. This gives a structure with 32 amino acid residues in the monomer or a dimeric structure of 64 amino acids. A second important feature of the structural design is the placement of the proline residues since this residue affects protein folding and determines the overall topology of the molecule. Analysis of a variety of known peptide sequences indicated that the sequence shown in Figure 6B will fold into the desired structure. A molecular model was constructed to confirm the folding structure, i.e., formation of β-pleated sheets, and other structural features including active site location.

The proposed betabellin molecule has been synthesized by solid phase peptide synthesis. The molecule is currently being purified by high pressure liquid chromatography. Preliminary characterization of crude betabellin fractions reveal that it has the expected molecular weight of about 7,000 daltons. Moreover, circular dichroism studies of the molecule's three-dimensional structure indicate that no α-helical domains exist, but a high degree of β-pleated sheet structure is present. Clearly, definitive structural analysis will require purification and crystallization of the molecule followed by x-ray diffraction studies.

Conclusion

The overall goal of the Molecular Biology Program is to elucidate the molecular basis of biological function. An understanding of the mechanism of complex biological systems such as the supramolecular structures described above will allow the manipulation of such systems for useful purposes. Ultimately novel catalysts, detectors, adhesives or sensors may be produced utilizing such knowledge and technology. Clearly such a capability will require insight into the detailed molecular aspects of biological systems and structure-function correlation.

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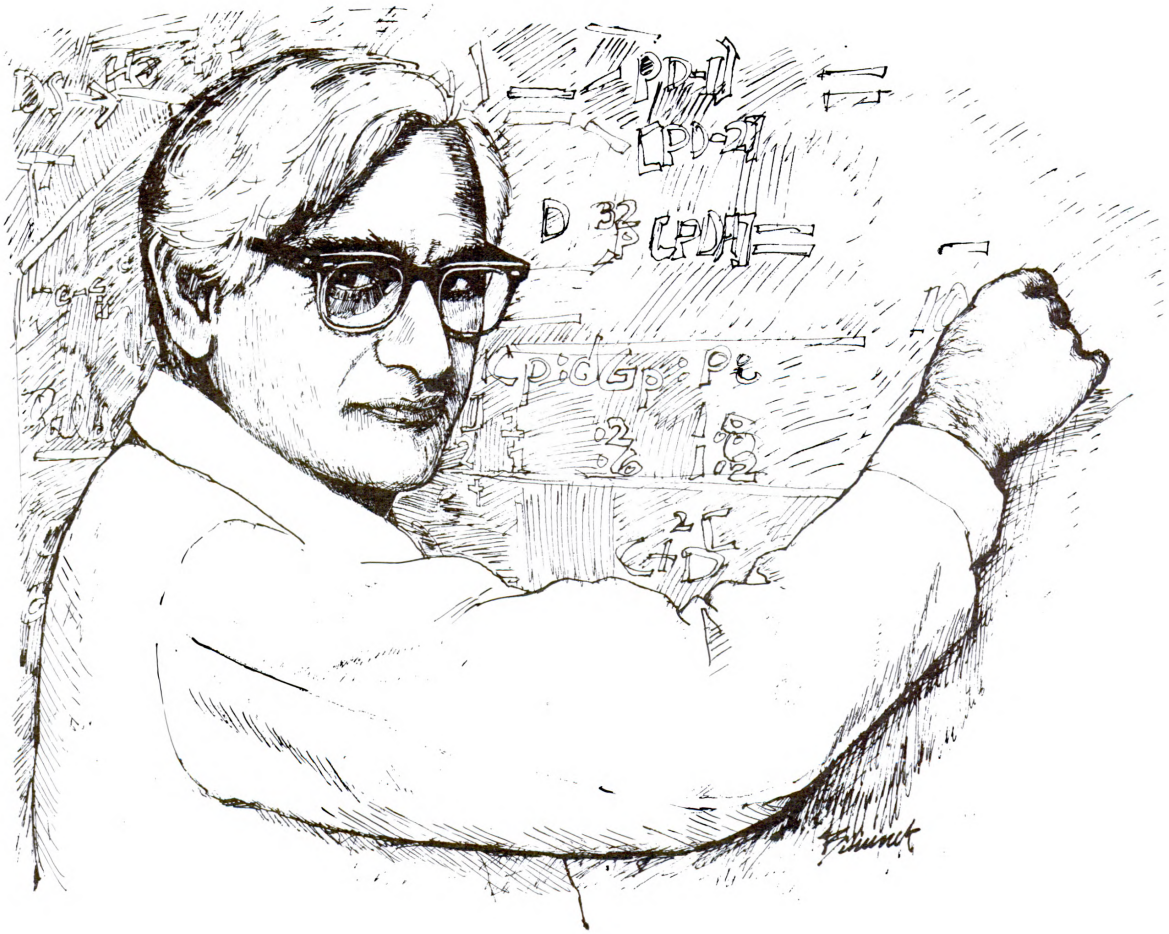
Dr. Schmell is the Program Manager for Molecular Biology at the Office of Naval Research. He is also a Visiting Associate Professor of Pharmacology and Experimental Therapeutics at The Johns Hopkins University School of Medicine. His current research interests include the biochemistry and immunochemistry of the human acetylcholinesterase.

Profiles in Science



PROFESSOR HAR GOBIND KHORANA is the Alfred P. Sloan Professor of Biology and Chemistry at the Massachusetts Institute of Technology and is a Principal Investigator of the Office of Naval Research studying the molecular and biological nature of vision. A better understanding of vision can be potentially useful in the design of operations and maneuvers by the Navy. Also, in view of a rather high prevalence of color blindness in the male population, the knowledge gained from Khorana's research may open up new ways to combat this defect.

Professor Khorana is renowned for his research and many publications (420) in the fields of organic, nucleic acid, and membrane protein chemistry. Using the methods developed by Professor Khorana, the problem of the genetic code was elucidated. His further work led to the total synthesis of genes in the laboratory. These methods are universally used in recombinant DNA studies. Using chemical cross linking reactions and recombinant DNA techniques, he is currently doing pioneering research on the molecular basis of light capture by biological systems (bacteria and mammals). This work is at the forefront of the field of biological light conversion.

In 1968, Professor Khorana shared the Nobel Prize in Medicine for deciphering the genetic code. Some of his other awards include the Lasker Foundation Award and the Louisa Gross Horwitz Prize; he is also a member of the National Academy of Sciences, U.S.A., the Royal Society, London, and the U.S.S.R. Academy of Science.

IMMUNOPHARMACOLOGY:

Medical Therapy of the Future

by Jeannine A. Majde
Office of Naval Research

The immune response has long been recognized for its essential role in resistance to infectious agents and cancer. Today it is known to participate in other types of diseases as well, not always as a friend. For instance, the immune response is the major villain in such common diseases as rheumatoid arthritis and asthma; it may even contribute to vascular disorders leading to heart disease and aging. Clearly, control of the immune response has great medical significance. As the response is unraveled by research, targets for manipulation within it become apparent.

Immunopharmacology can be defined as the use of synthetic drugs or biological products to alter the immune response, either in a positive or negative direction. The term has been in use for only about five years although drugs and biologicals have been used clinically to suppress inflammation and immunological rejection of tissue grafts for about two decades. The rapid expansion of immunology has defined other directions for immunopharmacological attack, and the field has grown accordingly. The International Society for Immunopharmacology was first formed in 1979; the Second Congress, held in July, 1982, had nearly 900 registrants. The pharmaceutical industry in this country, as well as in Japan and Europe, has recently developed a healthy appreciation for the commercial potential of immunopharmacology. In this report I will attempt to show the directions the field is taking and illustrate its future applications in military medicine.

Technical Background

The immune system is responsible for the elimination of foreign substances, or antigens, from the body. The specific immune response can be described in very simplistic terms as an interaction between two types of cells, macrophages and lymphocytes, after exposure to antigen followed by proliferation and differentiation of the lymphocytes to form effector cells.* These effector cells may attack the foreign substance directly (cellular immunity) or indirectly via antibody formation (humoral immunity). Cellular immunity is mediated by lymphocytes derived from the thymus (T cells) while humoral immunity is mediated by lymphocytes from the bone marrow (B cells).

A more primitive and faster acting response with the same basic function is termed non-specific resistance; it is non-specific because it requires no previous exposure to the antigen. This response is also mediated by the macrophage as well as by another class of "white" blood cell, the granulocyte. Macrophages and neutrophilic granulocytes phagocytize (ingest) foreign materials and digest them with intracellular enzymes. Recently a new cell type has been recognized in non-specific resistance—the natural killer or NK cell. This cell appears to be a lymphocyte that secretes enzymes. NK cells are activated by interferon and kill cancer cells and virus-infected cells on contact. Non-specific resistance mechanisms are not well understood but are of great interest to immunopharmacologists concerned with infectious diseases and cancer. This is because non-specific resistance is always in place; it does not require a lag period of five days to two weeks to act as does the specific immune response. Proper manipulation of non-specific resistance can prevent a microorganism (or cancer) from gaining a foothold in the body and is essential for "cleaning up" following therapy of such diseases. The macrophage appears to be the "controller cell" in both non-specific and specific resistance, and it has become the focal point of immunopharmacology.

While this summary ignores the elaborate interactions between lymphocytes involved in specific immunity and the elegant amplification mechanisms such as serum complement, it should serve to provide a vocabulary for what is to follow.

Categories of Immunopharmaceuticals

The field of immunopharacology is far too new (and burdened by commercial interests) to have arrived at a consensus regarding categorization of drugs such as the one I use in this article. Many of the concepts developed below are based on my own research with several of the drugs and on frank discussions with developers of those I have not used in my own models. Thus, the material in this section reflects my own experiences and biases, a fact that should be clearly recognized by the reader. With this caveat, I suggest that immunopharmaceuticals my be divided into three sub-categories: immunosuppressants, immunostimulants or immunopotentiators, and immunomodulators.

Immunosuppressants

The oldest branch of immunopharmacology has exploited the fact that the immune response requires proliferation of cells—drugs toxic for such proliferating cells suppress immunity. Unfortunately, this suppression is not specific for the particular immune response causing the problem (such as the tissue graft rejection response or an autoimmune disease) nor is it specific for cells mediating the immune response; all proliferating cells are blocked. Thus, the traditional drugs used to block the immune response cause generalized immunosuppression, resulting in greatly increased susceptibility to infections and cancer, as well as generalized destruction of proliferating cells in the bone marrow and lining the intestinal tract. The end result is very similar to radiation sickness; such serious side effects place severe limitations on the use of this cytotoxic approach. As our understanding of the interactions among cells required for the immune response increases, we are better able to engineer drugs more specific for a single ongoing immune response. Of great current interest in transplantation biology is a drug called cyclosporin A, a fungal product. This drug is somewhat more selectively toxic for recently stimulated lymphocytes. A radically different approach is the selective stimulation of some of the specific and non-specific suppressor mechanisms that have been found to be operative in endogenous control of the immune system. While no methods for activation of these suppressor mechanisms are now recognized, they hold great promise for both transplantation biology and the treatment of autoimmune diseases, in which these mechanisms appear to be defective.

Immunostimulants or immunopotentiators

A need for immunostimulants, or adjuvants, for promotion of immune responses to vaccines has been long recognized in the clinical arena. In experimental studies with animals, a crude mixture developed in 1935 by Freund is used. This mixture, which consists of mineral oil, an emulsifier, and killed tuberculosis bacteria (called Freund's complete adjuvant), is highly effective at stimulating a vigorous response; however, it causes chronic ulceration at the site of immunization and cannot be used in humans. The only clinically ac-

*A third class of cells, dendritic cells, have recently been implicated in antigen presentation to lymphocytes, but this component of the immune response is still in the beginning stages of research.

ceptable adjuvant is aluminum hydroxide; this salt is not toxic, but it is thought to selectively promote a form of immunity leading to allergy. In the last decade, investigators have succeeded in isolating and characterizing the active component of Freund's complete adjuvant from tuberculosis bacteria cell walls. This substance is a sugar-dipeptide called muramyl dipeptide (MDP). MDP does not exibit the local toxicity of Freund's; but it does induce fever, sleep, and autoimmune diseases. A number of chemical derivatives of MDP are now under development to eliminate these side effects.

Another class of immunostimulants is composed of living attenuated bacteria. The best example of this is called BCG, a strain of bovine tuberculosis. These bacteria have been used in cancer therapy but have caused serious side effects. Other immunostimulants have been derived from bacteria other than tuberculosis organisms, such as pertussis, *Corynebacterium parvum* and brucella organisms. To date, only crude extracts of the latter organisms have been studied which are quite toxic in this form.

Another class of immunostimulants are the more chemically defined polyanions. These are large polymers with a net negative charge. Examples are maleic anhydride-divinyl ether (MVE), dextran sulfate, and poly A:poly U. These molecules are potential interferon inducers as well as direct stimulants of macrophage functions; interferon acts as an immunomodulator (described in the following section) and thus these substances are not always predictably stimulatory. Their specific action is dependent on their molecular weight and the rate at which they are degraded in the host. Systematic studies with more uniform preparations are just now getting underway.

Another chemical class of immunostimulants are the polysaccharides—complex sugars obtained from fungi or bacteria. The most extensively studied member of this class is glucan (beta-1, 3-polyglucose), which has been used in clinical trials in cancer patients. Two sources of glucan have been used, one the baker's yeast *Saccharomyces cerevisiae*, the other the organism *Schizophyllum commune*. Another immunologically active polysaccharide is CPS-K, a polymannose from *Klebsiella pneumoniae*. These compounds have the disadvantage of being non-degradable by the macrophages they stimulate, which eventually blockades macrophage function.

Immunomodulators

The natural immune response is tightly regulated by certain classes of lymphocytes as well as by macrophages. Once initiated, its suppression is programmed and predictable in the normal host. If natural immunosuppression fails to occur, autoimmune diseases may result. Compounds properly termed immunomodulators do not override these natural immunosuppressive signals (as can happen with immunostimulants) and therefore are less likely to cause a pathologic immune response. Thus, immunomodulators may suppress or stimulate the immune response depending on the physiological state of the host. Two broad categories of immunomodulators are recognized: natural biologics and synthetic drugs. Although the immunostimulants discussed above are often termed immunomodulators, I use the term for substances that up- or down-regulate the immune response depending on the physiological state of the host rather than depending on the timing of administration of the substance relative to the antigen. My discussion of immunomodulators will focus on the two main sources—natural biologics and synthetic drugs:

Biologics: Biologics that serve as immunomodulators are termed cytokines or leukokines, i.e., glycoprotein regulators secreted by macrophages (monokines) and lymphocytes (lymphokines). Few cytokines have been chemically defined and they are studied primarily with somewhat ambiguous bioassays. Intensive work is underway to purify and define the cytokines. Some of the more distinctive cytokines are the interleukins (lymphocyte activating factor, T cell growth factor, B cell growth factor) and the interferons. The latter are the best defined cytokines, being purified in alpha, beta and gamma forms. The interferons were originally defined by their capacity to induce cellular resistance to viruses, but it is now known that they have many effects on cell growth and immune function. Gamma-interferon is now thought to be identical to macrophage activating factor. Many advances are expected in the cytokine field in the near future. Their value as therapeutics is unknown at present, and it is likely that their utilization will require some form of encapsulation delivery system to permit their escape from biodegradation.

Synthetics. While advances in bioengineering permit the prospect of synthesizing large quantities of immunologically active biologics at a reasonable cost, synthetic immunomodulators are likely to remain the most economical and convenient approach to immunotherapy in the foreseeable future. A wide variety of synthetic compounds have been found to have immunomodulatory activity, and in most cases this activity has been found serendipitously. A notable exception is a group of compounds developed in Japan by Professor Hamao Umezawa, Director of the Institute of Microbial Chemistry, Tokyo (who discovered kanamycin, neomycin, etc.). These compounds, the best known of which is bestatin, all act by inhibiting cell surface proteases that appear to be active in development of the immune responses. Other true synthetic immunomodu-

lators that have received extensive study and illustrate the broad chemical profile associated with immunomodulatory activity are:

1. Isoprinosine or inosiplex—an ionic complex of the purineinosine and an alcohol, which was developed in the U.S. Isoprinosine was originally developed as an antiviral, and then was found to manifest its antiviral activity by potentiating the immune response. The drug is orally active and non-toxic in massive doses, although it causes mild euphoria and is metabolized to uric acid causing gout in susceptible individuals. It is distributed in numerous countries as an antiviral without prescription but has not been approved for use in the U.S. except for treatment of a lethal measles infection of the brain (subacute sclerosing panencephalitis) with special permission. Recently it has been lauded in France for its therapeutic value in immunosuppressed cancer patients suffering from herpes zoster (shingles) and mild activity has been seen in other herpes virus infections. The efficacy of the drug is not easy to demonstrate with conventional disease models, and it is likely that its best application will be in combination with other drugs, including conventional antibiotics and interferons. As with the drugs discussed below, Isoprinosine has a more pronounced effect in stressed animals.

2. Levamisole—an imidazole anti-helminthic (worming) drug also found by accident to be an immunomodulator. This compound has received extensive trials in the U.S. in cancer patients and viral diseases, but its efficacy is so limited that it has fallen out of favor. It has also been used in rheumatoid arthritis with equivocal results. Unlike Isoprinosine, Levamisole causes some bone marrow toxicity, central nervous system (CNS) symptoms and gastrointestinal problems.

3. Therafectin or SM-1213—a non-metabolizable cationic glucose, which has received limited study in man, but animal studies demonstrate broad-spectrum anti-microbial efficacy at extremely low doses (as little as 1 mcg/kg) and anti-inflammatory activity at 'high' doses (15 mg/kg). The compound is orally active (although parenteral administration may be more efficacious) and non-toxic in normal individuals.* Clinical trials are being considered in rheumatoid arthritis and AIDS (acquired immunodeficiency syndrome) patients. Animal studies suggest that this compound requires a "stress signal" for activation that appears to be provided by a disease state but may be absent in experimental systems in healthy animals.

4. CCA (N-(2-carboxyphenyl-4-chloroanthranilic acid disodium salt)—a compound developed in Japan which demonstrates a similar broad range of anti-microbial activities but has been studied primarily as an anti-inflammatory in immunologically driven inflammatory diseases such as rheumatoid arthritis. Discussions with its developers at Chugai Pharmaceuticals in Tokyo revealed that its behavior in anti-microbial models parallels that of Therafectin in that environmental conditions appear to influence the results, perhaps via stress hormone signals.

5. CP-20.961 (a lipoidal amine)—this compound might be more appropriately placed in the immunostimulant class as it has good adjuvant activity; but when used as a stimulator of non-specific immunity, it shares many of the biological features of the immunomodulators discussed above. When given prior to infection, it increases mortality rather than decreasing it. Several immunomodulators appear much more protective when administered after the infection is established.

The drugs briefly discussed above are those that have received the most study, and they illustrate the rather unexpected range of chemicals that manifest similar immunomodulatory activity. It might even be appropriate to include ascorbic acid (vitamin C) in this list although equivalent studies focusing on the immune system have not been reported with this controversial compound. A number of new derivatives of MDP behave more like immunomodulators than immunostimulators in that they do not appear to override immune regulatory mechanisms, at least to the same degree as the parent compound. Such compounds are being intensively studied by drug firms in Europe, Japan and the U.S with the focus on adjuvant activity for vaccines. It is far too early to predict the ultimate safety and efficacy of the MDP derivatives as adjuvants or as inducers of disease resistance.

It is important to realize that immunotherapy with stimulants and modulators represents a radical departure from conventional drug therapy which is based on an antibiosis principle, i.e., inhibiting the causative agent of the disease, either a microbe, an enzyme or a tumor. Stimulatory immunotherapy instead operates through activating natural immune functions of the host and should be thought of as "pro-biosis" or "pro-host" in action. Effective use of pro-host drugs requires a much better understanding of the host defense mechanisms and endogenous regulatory mechanisms being altered than is now available. In fact, many of the immunomodulators might more reasonably be termed immunonormalizers in that they appear to function by restoring the immune response suppressed by a disease state or other stress to 'normal' levels. The need for very basic research in these broad areas is apparent.

*A category of individuals insufficiently studied with respect to immunomodulators are those suffering autoimmune diseases. Much work is needed in the excellent animal models of such diseases to determine if these compounds are harmful or beneficial in unbalanced immune conditions.

Applications of Immunopharmaceuticals to Military Medicine and the ONR Program

As implied in the discussion above, two broad applications of stimulatory immunotherapy to military situations are apparent:

Adjuvant Development

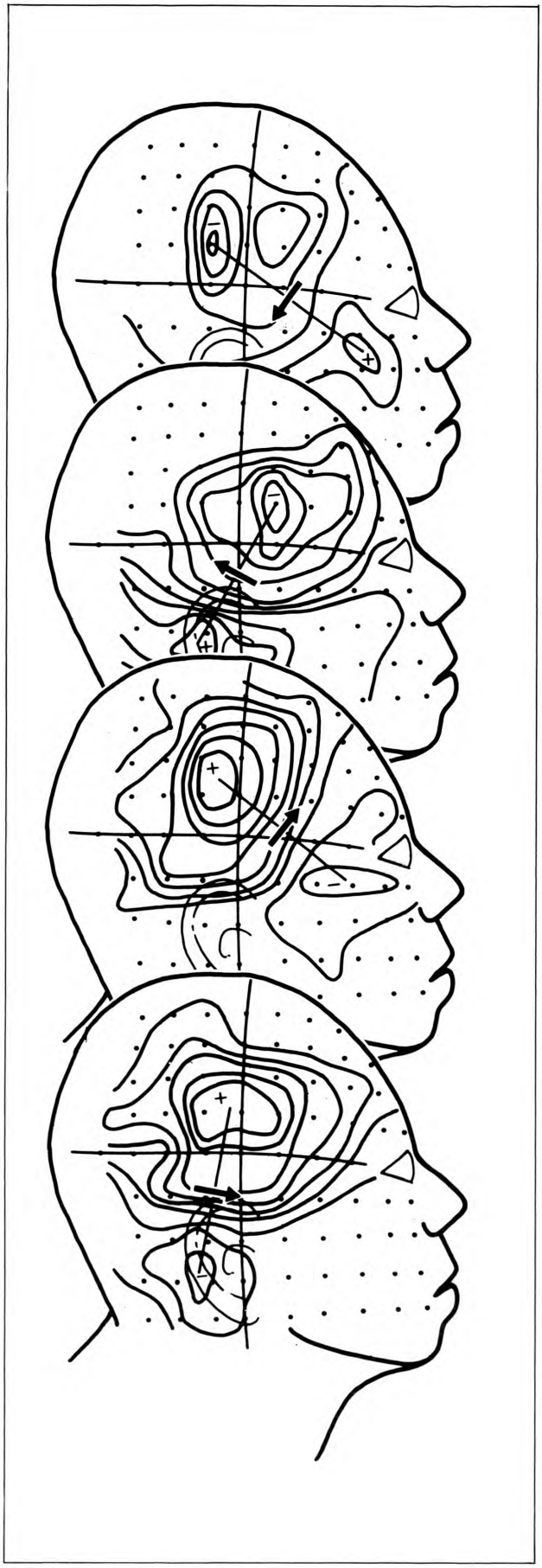
Military populations are particularly prone to epidemic diseases that can be predicted and prevented by vaccination. While many of the standard vaccines produce protective immunity without the addition of an adjuvant (largely because they contain microbial products with natural adjuvanticity or else contain living agents that produce a subclinical disease, and thus induce natural immunity, e.g. Sabine polio vaccine, smallpox vaccine), new vaccines are under development that are highly purified materials with low intrinsic immunogenicity. These vaccines are ultimately safer and more economical to produce but will require an adjuvant to be effective. An ongoing research program at the Office of Naval Research (ONR) is exploring the potential of both natural cytokines and synthetic immunostimulants as adjuvants for poorly immunogenic vaccines. Some clear directions for development of a clinically safe and efficacious adjuvant are anticipated by 1986.

Stimulation of Non-Specific Resistance

While active immunization with a vaccine is the optimal approach to preventing infection with defined pathogenic microorganisms, development and production of a vaccine for each potentially infective organism (as is required by the intrinsic specificity of a vaccine) is impractical. Vaccine development takes years to decades and costs several millions of dollars; production of vaccines is also very costly. An equally important limitation of the vaccine approach is the fact that new organisms are constantly being discovered (Legionnaire's disease and AIDS are current examples) and genetic engineering can generate novel antigenic forms for use in biological warfare for which no vaccine can be made. A potential solution to this major problem is the use of immunopharmaceuticals with the capacity to stimulate the natural defense systems of the potential victim to levels of competency that will prevent or ameliorate the infection. These natural defense systems routinely eliminate millions of microorganisms encountered in our daily lives and, as mentioned earlier, form the first line of defense against infection. Fortunately, most of the organisms for which we have no broad-spectrum antibiotics are intracellular pathogens such as viruses—their ultimate survival depends on evading microbicidal mechanisms of macrophages and granulocytes or non-specific resistance factors such as the interferons. Drugs that greatly potentiate these non-specific resistance mechanisms or protect them from stress-induced suppression, or both, may be a very workable solution. The known drugs with this capability, such as isoprinosine, have low efficacy. A new MDP derivative, called MTP-PE, appears to have very high efficacy; a single administration of the compound to the nasal membranes prevents a respiratory viral infection for up to two weeks and has therapeutic activity up to one week following the infection. While many drug firms are pursuing immunomodulator research in Europe and Japan in the context of cancer therapy, relatively little has been done with infectious diseases. In large part this is because infectious disease work is arduous and expensive to undertake. A serious deficiency of the work to date in both cancer and infectious agent models is that it has focused on optimizing route, dosage, etc., and little effort has been devoted to mechanism of action of the drugs. The ONR immunology program is attempting to perform systematic comparisons of the known compounds using infectious models and to probe their mechanisms of action at the cellular level. An ONR program planned for the near future will examine molecular mechanisms and attempt to develop a rational approach to design of safe and efficacious drugs. Another ONR program being proposed will be directed at the hormonal regulatory mechanisms that control immune function in order to define directions for pharmocologic manipulation. This three-pronged attack on the general problem of enhancing non-specific resistance offers a realistic hope that the threat posed by infectious diseases to operational forces can be significantly reduced.

Author

Dr. Majde is the Scientific Officer for Immunology and Acting Program Manager of Cellular Biosystems at the Office of Naval Research. Her published papers and research have been primarily in the fields of virology and immunopharmacology. She is also Adjunct Research Professor in the Department of Biochemistry at The George Washington School of Medicine.



Magnetically Localizing the Sources of Epileptic Discharges within the Human Brain

by

Jackson Beatty, Daniel S. Barth, and William Sutherling
University of California, Los Angeles

Understanding how the human brain works—both in health and in disease—is the most complex problem in all of biology; a problem made all the more difficult by the bony fortress of the skull that shields the brain from harm. Because of the skull, we can rarely observe the human brain directly. Instead we must be content with the limited visions of the brain provided by a variety of brain imaging techniques, each of which emphasizes a different aspect of the brain and its physiological activity.

New brain imaging techniques arise as advances in the physical, chemical, and engineering sciences are exploited to reveal new and unique aspects of brain function and structure. Among the most recent of these developments is neuromagnetometry. Made possible by advances in low temperature physics, the neuromagnetic image of the brain maps the magnetic fields sensed at the scalp that are produced by the electrical activity of brain cells. From such maps, intracranial flows of current may be studied.

Neuromagnetic measures have made their most important neurological contribution in the study of focal epilepsy, a disorder characterized by sudden, massive electrical discharges within the brain. Such discharges may be localized by measuring the magnetic fields that they generate and so provide guidance for surgical treatment.

Brain Magnetic Fields

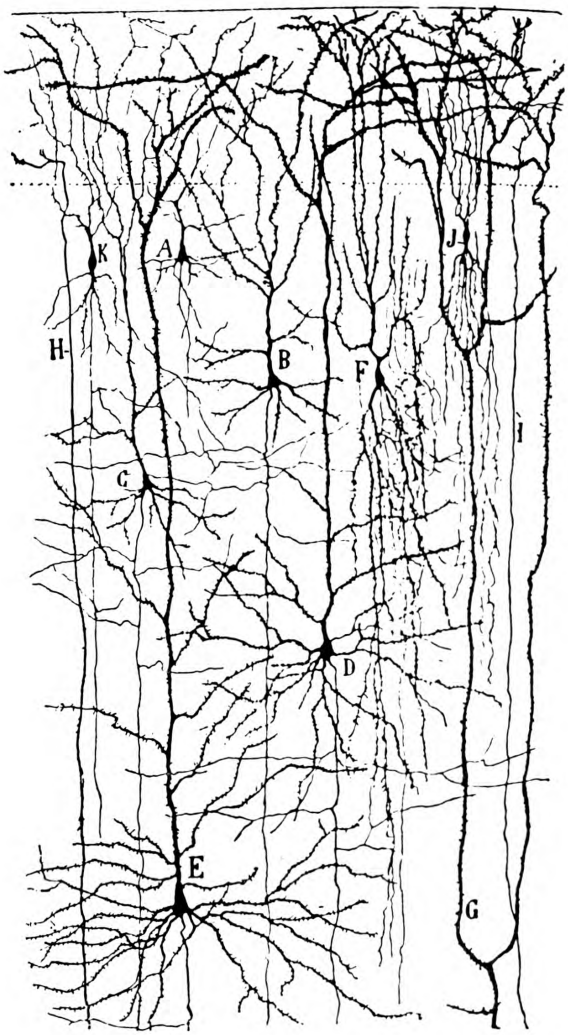
The magnetic and electrical fields generated by both normal and epileptic brain tissue are produced by nerve cells or neurons. It is the neurons that carry information from the sense organs to the brain, that process information within the brain, and that issue commands to the muscles of the body by way of the motor nerves. They accomplish these feats in part by encoding information as variations in electrical potential across their outer membranes, which separate the interior of the cell from the surrounding electrolyte rich fluids. Thus, information may be transmitted from one region of a neuron to another by initiating a flow of current within the neuron as an electrical signal. Such electrical signals must also produce magnetic fields, although in many circumstances the magnetic field generated by electrical events within a single neuron is exceedingly small.

Under certain conditions, the miniscule magnetic fields produced by single brain cells may summate to produce a magnetic field that can be recorded at the scalp. For this to occur, the contributing neurons must be both large and asymmetric. Larger cells are capable of conducting intracellular currents over longer distances and therefore can produce a larger magnetic field. Asymmetric cells produce magnetic fields that are not self-cancelling. Figure 1 illustrates an important class of cells that meet both of these requirements, the pyramidal cells of the cerebral cortex. It is the pyramidal cells that are thought to contribute most heavily to the recordable magnetic fields of the brain.

Another condition for summation of magnetic fields is that the contributing cells be oriented similarly within the brain; the pyramidal cells of the cortex also fulfill this requirement. Finally, for summation to occur, the contributing neurons must be functioning in unison, which certainly is the case during epileptic discharges.

Figure 1

This classic drawing by the Nobel Prize winning neuroanatomist Santiago Roman y Cajal illustrates the pyramidal cells of the cerebral cortex that may serve as current dipoles generating neuromagnetic fields. Pyramidal cells of different sizes are indicated by the letters A, B, C, D, and E. During activation of these cells, current flows between their triangle-shaped cell bodies and the long dendrite that extends upwards to the surface of the cortex. Notice that these pyramidal cells are precisely aligned so that their magnetic fields may summate.



One very fortunate characteristic of the magnetic field produced by the activation of a local population of neurons is that it is restricted to the region of its origin. There are several reasons why this is the case. First, it is the high density, highly localized intracellular current that generates the measured magnetic field; under many conditions the contribution of the widely dispersed extracellular return currents is negligible. Second, any magnetic field is concentrated about its origin since the strength of the magnetic field decreases with the square of the distance from its source. Finally, the skull is transparent to magnetic fields so that it does not smear or distort the field patterns produced by brain tissue.

The magnetic fields associated with many epileptic events are orderly in their structure; the current flow that gives rise to the magnetic fields recorded under normal circumstances may be modelled as a simple current dipole. The magnetic field of a current dipole describes circles around the source which at the scalp are measured as magnetic flux emerging from the head on one side of the source and reentering the head on the other side of the source. The field obeys the "right-hand rule" of elementary physics: for a current source oriented in the direction of the thumb of the right hand, the resulting magnetic field will circulate in the direction of the curled fingers. The electrical field should ideally be of opposite polarities at the two ends of the source. Figure 2 illustrates the orientation of both electrical and magnetic fields about a current dipole. The magnetic field recorded in epilepsy often approximates this pattern.

Brain magnetic fields are very weak, particularly compared with typical magnetic disturbances produced by non-biological sources in the environment. This may be seen in Figure 3. Magnetic field strength is best described in terms of flux density. The standard unit of flux density in the Systeme International (SI) is the tesla. The tesla is a secondary unit equal to 1 newton/ampere-meter, where a newton is equal to 1 kilogram-meter/second squared.

The flux density of brain magnetic fields is on the order of 0.01-1 picotesla. This is two orders of magnitude smaller than the fields produced by skeletal or cardiac muscle, six orders of magnitude smaller than the fields produced by large moving metal objects such as automobiles and elevators, and eight orders of magnitude smaller than the magnetic field of the earth. These considerations pose two requirements for the measurement of the magnetic fields of the brain: first, the measurement system must be very sensitive and, second, a substantial signal-to-noise ratio problem must be solved.

Figure 2

Theoretical distributions of electrical and magnetic fields produced on the surface of the head by a tangentially oriented current dipole within the brain. Pyramidal cells have such an orientation along the fissures of the brain.

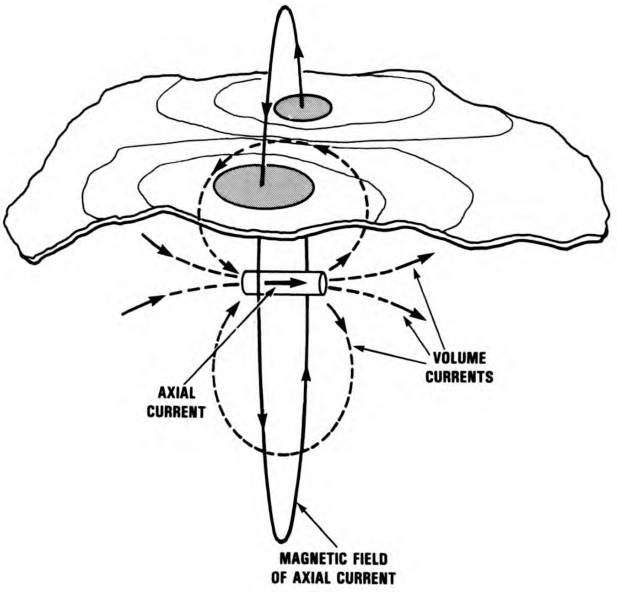
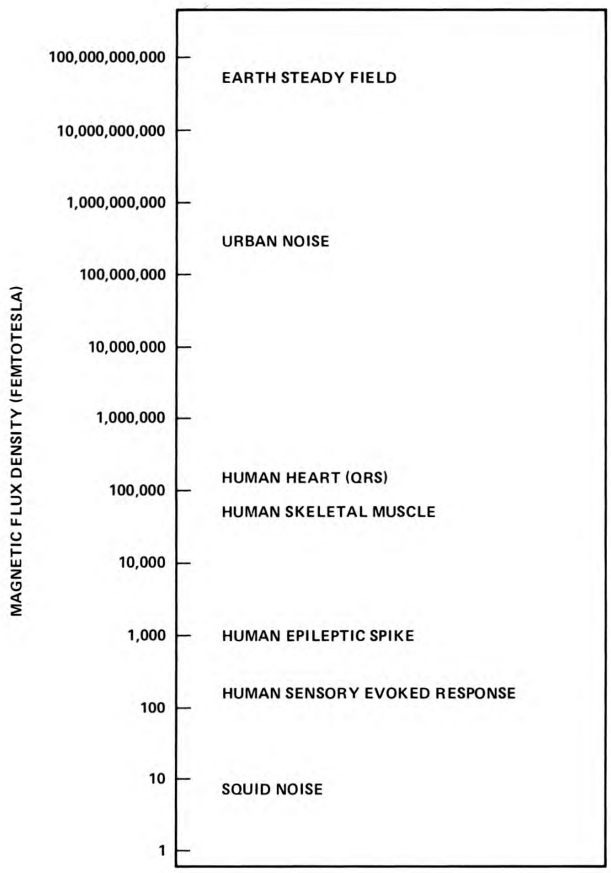


Figure 3

The strength of biological and environmental magnetic fields.



Neuromagnetic Measurement

The reliable measurement of the weak magnetic fields of the central nervous system was made possible by the development of a highly sensitive magnetic sensor called the superconducting quantum interference device (SQUID) developed by James Zimmerman, of the National Bureau of Standards and colleagues in 1970 with the support of the Office of Naval Research. The SQUID may be used to sense magnetic fields directly or be coupled to specially designed superconducting detection coils. Perhaps the simplest configuration for the detection coil is a single loop of wire. Although very sensitive, such a design is incapable of rejecting environmental noise and must therefore be operated in magnetically shielded rooms or in remote locations. The input coils can also be given more complex configurations that cancel magnetic noise. One that is widely used is that of a second-order gradiometer, a concentric set of coils sensing the second spatial derivative of the field gradient traversing them. This configuration makes the gradiometer selectively sensitive to very close sources relative to distant sources, thereby achieving a high degree of rejection of background noise. Gradiometers are practically insensitive to uniform fields such as the large field of the earth, a selectivity that is achieved at some cost in the overall sensitivity.

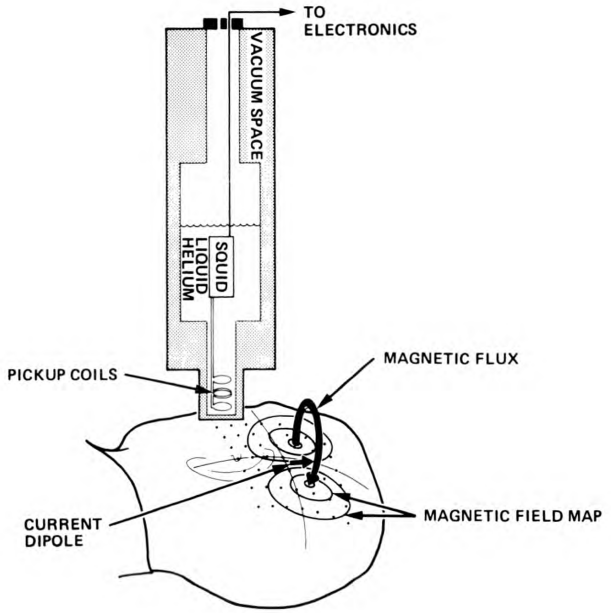
In a superconductive gradiometer, the flux transporter and SQUID are mounted in a superinsulated dewar filled with liquid helium, keeping the sensing apparatus at 4.2 Kelvin. The dewar (about 1.2 m in length) is mounted on a non-metallic support structure that allows its precise positioning over specific points on the subject's scalp (see Figure 4).

The commercially available magnetic recording systems utilize a single-channel sensor that is coupled to a gradiometer configured to record the component of the field that is normal to the head. The output of the SQUID control circuitry is directly proportional to the gradient of magnetic flux. In a typical recording session, the gradiometer is sequentially positioned through a matrix of closely spaced points thought to encompass the entire extracranial magnetic field produced by a given neural event. This matrix of points is referenced to skull landmarks. Signal averaging is used to improve both the signal to noise ratio and the statistical reliability of the signal. The amplitude of the time series at a particular latency is proportional to the instantaneous magnetic flux density at the recording point. The polarity of the time series represents the direction of the field lines at that point, either entering the head or emerging from the head.

The recordings from each point in the matrix are later reassembled by computer into a topographical map representing the spatial distribution of the amplitude and polarity of the magnetic fields at selected points in

Figure 4

In recording brain magnetic fields, the neuromagnetometer probe is sequentially positioned at 2 cm intervals along a premarked recording matrix. The probe itself consists of an insulated dewar containing liquid helium, cooling the pickup coils and the SQUID to superconducting temperatures.



time. A magnetic field map of an area on the scalp is considered complete when it reliably displays symetrical field maxima of opposite polarity where the flux emerges from and reenters the head and when the strength of the field approaches zero at all borders of the map.

Localization of the intracranial source capable of producing a particular magnetic field pattern depends both upon the measured properties of the field and upon the model assumed for the generating source. The simplest and presently most useful model is that of a current dipole; here the orientation of the underlying source is orthogonal to a line connecting the two field maxima and its surface location is exactly midway between the maxima. The direction of the current may be determined by applying the right hand rule to the direction of field circulation.

Finally, the depth of the source may be calculated from the distance between the maxima and by making certain assumptions about the volume containing the source. The field pattern produced by a current dipole embedded in a finite conducting volume depends on the geometry of the volume. One of the most useful models of brain geometry is the spherical model. The model of the current dipole in a conducting sphere has generally produced depth estimates that are consistent with general neuroanatomical data about the most likely locations of individual sources in the cortex. It also should be noted that concentric variations in conductivity in a sphere, such as the skull, do not affect the pattern of the external magnetic field. However, any serious deviations from the spherical model may affect the localization derived from the observed field pattern.

The Problem of Epilepsy

Human epilepsy is a disorder characterized by the abnormal electrical discharge of nerve cells within the brain, resulting ultimately in behavioral seizures. Of the variety of seizure disorders that have been recorded in man, focal (partial) epilepsy is the most common. It is estimated that in the United States alone, nearly 800,000 individuals have focal epilepsy. Furthermore, in as many as 360,000 of these patients the epilepsy is not controlled by medication; surgical removal of the epileptic brain tissue usually associated with a scar is estimated to be an effective treatment in about 15 percent of these cases.

In focal epilepsy, the abnormal brain tissue responsible for seizures is generally restricted to a discrete region, called the epileptic focus. Periodically, neurons within the epileptic focus lose their normal regulatory control and begin a massive synchronous discharge. This focal seizure disrupts the normal functioning of the brain within the region of the epileptic focus. The result

is a focal or restricted seizure. In some cases, this disruption spreads to other areas of the brain, producing a generalized seizure. The defining characteristic of focal epilepsy is that the seizure-instigating epileptic tissue usually is confined to a limited area and is not diffusely distributed throughout the brain. It is this fact that makes surgery a possible treatment for focal epilepsy.

The measurement and localization of abnormal epileptic activity within an epileptic focus is of major importance to neurosurgical therapy since accurate localization of the damaged tissue is the necessary first step in surgical planning. But the study of the epileptic focus is also of great interest to neuroscientists, who wish to understand the physiological mechanisms underlying seizures. Historically, investigations of focal epilepsy have made substantial contributions to our knowledge of the brain; for example, the first indication of cortical specialization was provided by the behavioral examination of focal epileptic seizures.

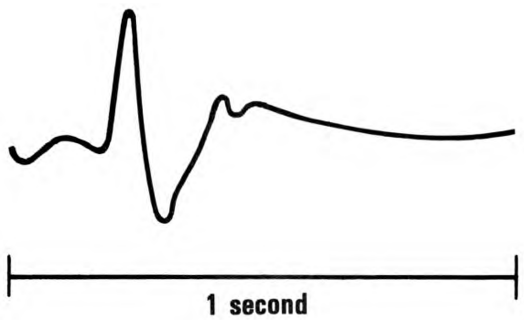
Interictal Epileptic Spikes

Many patients with focal seizure disorders display randomly occurring, transient interictal discharges ("ictus" means seizure). These interictal spikes are brief, high voltage, cellular discharges with a characteristic spike and wave appearance that is shown in Figure 5. Interictal spikes may be recorded electrically over wide regions of the scalp using the familiar electroencephalogram (EEG). Comparison of interictal spikes recorded in the scalp EEG to those detected by electrodes surgically implanted in or placed directly on the surface of the brain have revealed that the electrically resistive skull greatly distorts and attenuates many signals. It is often difficult to determine with precision the sources of either interictal spiking or seizure onset from scalp EEG data alone, hence the use of surgically implanted electrodes to localize both the source of interictal spiking and seizure activity.

We have utilized neuromagnetic recording, in conjunction with conventional EEG recording, to determine the intracranial locations of the sources of interictal spiking in a series of patients with focal epilepsy. Viewed magnetically, the intracranial location of the discharging epileptic tissue is apparent. Figure 6 shows the magnetic fields produced by interictal discharges in the right temporal lobe of a young male with focal epilepsy. Panel 6A shows the matrix of recording points from which neuromagnetic measures of interictal spiking were obtained. The actual averaged magnetic fields produced by the interictal discharges are shown in panal 6B. This classical interictal spike pattern is marked by a biphasic discharge, indicated here as components 1 and 2. A careful inspection of these data indicates that the polarity of these neuromagnetic events reverses across the recording field; this reversal illustrates the emerging and entering magnetic fields produced by each component. The magnetic field maps of both components are shown in Figures 6C and 6D, respectively. These maps are indicative of a localized current dipole discharging at a depth of approximately 2 cm below the scalp in the inferior region of the right mid-temporal lobe of the brain. Similar cases of a unitary cortical generator of interictal spike and wave discharges have been seen in approximately one-half of the epileptic patients examined neuromagnetically in our laboratories at UCLA.

Figure 5

A typical interictal spike recorded from the scalp.



Complex Discharge Patterns

Not all interictal spikes are as simple as those shown in Figure 6; evidence obtained from surgically implanted electrodes frequently indicates that the interictal spike actually forms a spike complex with different, neighboring regions of cerebral cortex discharging repeatedly in a stereotyped sequence. These more complex intracerebral epileptic events may also be imaged neuromagnetically. Figure 7 illustrates the more complicated series of discharges produced in the brain of another young male patient. Here two different sources, labeled A and B, generate biphasic discharges, producing four separate temporal components in the magnetic field maps. The first reflects the initial discharge of source A in the right anterior temporal lobe. The second component, occurring 16 milliseconds after the first, reflects the first discharge of source B, produced in a neighboring region of the temporal lobe, but displaced by 1 cm behind and below A. The third component is

Figure 6

Magnetic field map of a single source underlying the interictal spike. (A) Matrix of measurement points (2 cm spacing) positioned over the right anterior temporal lobe. (B) Averaged magnetic spikes from each point in the measurement matrix revealing a spike and sharp wave (components 1 and 2) reversing polarity between the top and bottom of the matrix. (C) and (D) Magnetic field maps for each of these components reveal a common location of the field maxima for each, indicating a source in the lateral right anterior temporal lobe. The polarity of the source (small arrows) is determined from the direction of field circulation ("+" for emerging and "−" for reentering) and reverses between the components. (fT = femto Tesla).

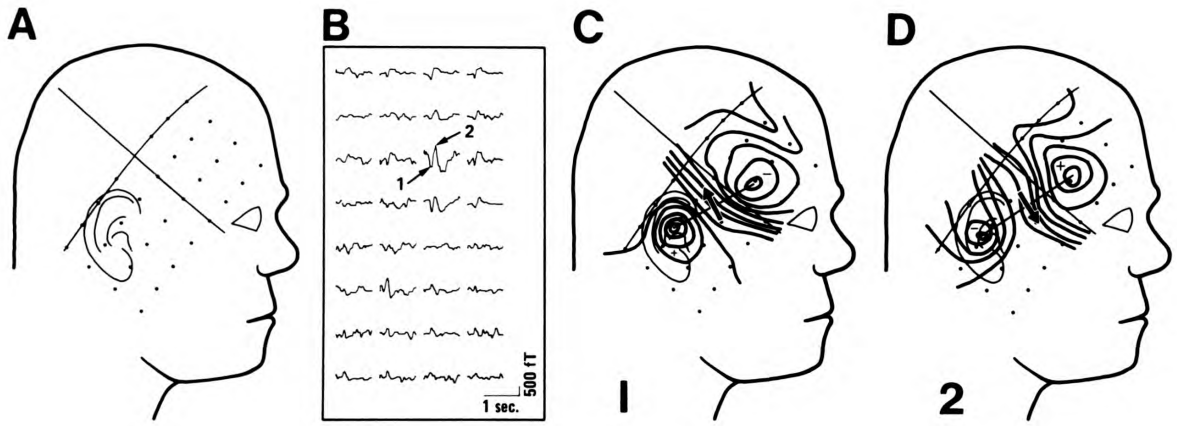
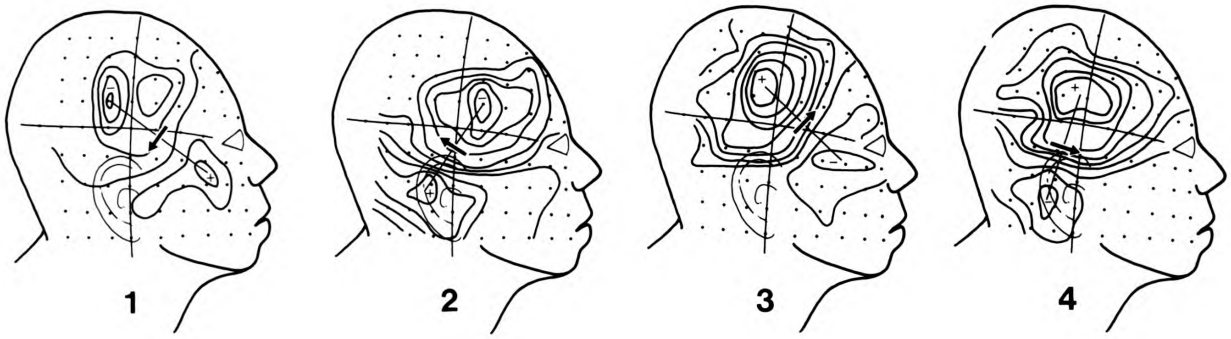


Figure 7

Sequential magnetic field maps of the interictal spike complex reveal a discharge pattern between two sources in the right temporal lobe of this subject. The discharge pattern consists of four temporal components, beginning with a spike in the anterior temporal region (1), followed by a spike in a second source 1 cm posterior and inferior to the first (2), and concluding with sharp waves in the first and second sources respectively (3) and (4).



the second discharge of source A, and the last component is the second discharge of source B. Viewed magnetically, it is often possible to unravel the complex spatial and temporal discharge patterns of sequentially discharging epileptic foci.

In this particular patient, the computed intracranial locations of the interictal discharges make anatomical and pathological sense. Both are situated at the edges of a large region of scar tissue within the right temporal lobe. Figure 8 shows x-ray computerized tomography scans upon which the computed intracranial discharge sources are indicated. The tissue within this scar probably contains few living neurons; therefore, it would not be possible for epileptic discharges to originate within the scar. However, at the borders of scarring, functioning neurons are often disturbed and irritated, producing intermittent epileptic discharges. Thus, the complex discharge pattern observed magnetically in this patient appears to result from irritative disturbances produced by a brain scar within the temporal lobe. These data, like those obtained from other patients, provide evidence that magnetic recording can localize the intracranial sources of abnormal activity within the epileptic human brain.

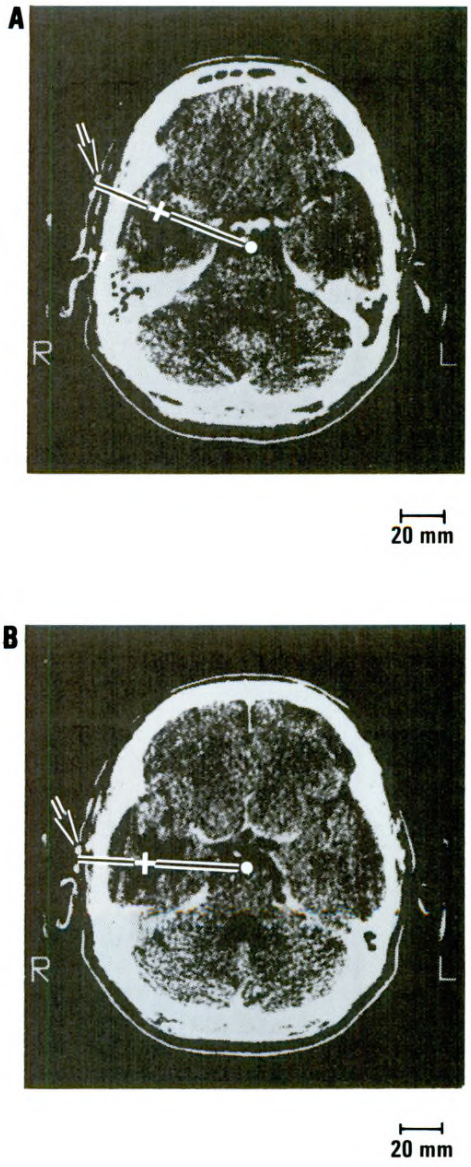
Future Developments

In the six years since Williamson, Kaufman, and Brenner wrote the first review of brain magnetic fields to appear on the pages of *Naval Research Reviews,* (30:10, October 1977, pp.1-18), a great deal of information concerning the brain and its functions has been obtained by neuromagnetic measurement. Clinical neuromagnetometry was conceived and entered its infancy during this half decade. But technically, neuromagnetometry has changed little during those years; only the introduction of a new type of SQUID detector with improved sensitivity has altered the technological picture portrayed by Williamson and his colleagues.

But many innovations are about to appear. The first multiple channel neuromagnetometer is being constructed and is expected to enter service within the year. Advances in cryogenic coolers offer the possibility of multiple, miniaturized magnetic probes that may be positioned about the skull to coax the brain into revealing its secrets. These are exciting possibilities. They will provide the technical advances needed to record and map the seizure activity of the brain, instead of just the interictal spikes that appear between seizures. The availability of multiple sensors is a necessary prerequisite to transform clinical neuromagnetometry from an advanced research method into a practical neurological procedure that may be performed in hospitals across the

Figure 8

CT scan sections taken at the levels of both sources display the position of underlying cortical structures. The locations of the first (A) and second (B) source (crosses) are plotted along radii connecting the center of the cranium with their respective surface locations marked with an aluminum washer attached to the scalp (arrow). The depth of both sources was approximately 3 cm, placing them along the lateral edge of a large area of scar tissue in the right temporal lobe.



nation. But perhaps most important is the possibility that multiple, simultaneous measurements of brain magnetic activity will make evident clues about brain function and disfunction that cannot be anticipated in advance of the use of the instrument.

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Research Notes

Microwaves Ease Withdrawal Symptoms of Morphine-Addicted Rats

Professor A.W. Guy of the University of Washington reports that his research program funded by the Office of Naval Research shows that low-level microwave fields significantly reduce withdrawal symptoms experienced by rats addicted to morphine. Rats exposed for 45 minutes to 2450 MHz fields of 1m W/cm^2, one and one-half days after withdrawal is initiated, exhibit attenuation in withdrawal syndromes such as "wet-dog shakes." These studies, coupled with others currently underway by the group, provide evidence that microwaves activate endogenous opioid mechanisms in test animals. Endogenous opioids are neuropeptides that, among other things, mediate stress responses. These studies are consistent with earlier observations by the same group that microwaves enhance the lethality of morphine. While at first glance these two findings may appear contradictory, it is quite likely that the same process that reduces withdrawal symptoms when morphine is absent will also enhance the effectiveness of morphine when it is present.

(Michael T. Marron, ONR).

New Evidence for the Molecular Basis of Higher Pressure Neurological Disorders

Many studies on the action of anesthetics under hyperbaric pressure and on the pressure effects on macromolecules and model membrane systems indicate that pressure can act directly on cell membranes. Such effects presumably alter the functional state of the membrane, including its receptor molecules for neurotransmitters, and may be responsible for the neurological disturbances observed in human subjects exposed to pressures equivalent to 500 feet sea water or more. Recently, Dr. Richard Taylor (A.D. Little, Inc.) has reported the results of his work on characterizing the acetylcholine receptor of rat skeletal muscle during pressurization. The rationale behind this approach was to discover if the binding of acetylcholine (the neuromuscular transmitter) is affected by conformational changes in the receptor, thus providing a possible explanation for the muscular tremors and other phenomena seen in high pressure environments.

The receptor was isolated either as a purified protein or as a combined proteoglycolipid. With both receptor preparations, acetylcholine binding was reduced by approximately 90 percent or more at 800 psi, well below the maximum pressures human subjects have experienced. The binding was recovered upon decompression, although complete renewal of the binding activity was observed only with the purified protein preparation. These data are the first to show the apparent alteration of the conformation of the acetylcholine receptor during compression, and the work leads the way in discovering the molecular bases for the incapacitating neurological derangements which occur in hyperbaric operations.

(Franklin G. Hempel, ONR)

Auditory Organization in the Human Brain

Professors S.L. Kaufman and S.J. Williamson at New York University recently reported the first noninvasive demonstration of a tonotopic organization in the human brain in response to steady state tones. They used a SQUID gradiometer to detect the magnetic fields associated with stimulation. In an extension of this work, tone bursts rather than constant tones were used; and it was found that reponses to the onset of the tone burst or to a long pulse originates in a second quite distinct region of the brain. This provides evidence for the existence of more than one auditory area in the brain, apparently with different functions. The possibility that there may be areas specialized to respond to speech-like features of acoustic stimuli in the human auditory cortex will be explored in the near future.

(Donald P. Woodward, ONR)

ONR Principal Investigator Receives Lasker Award

Dr. Eric Kandel, of Columbia University, is one of five researchers to receive the 1983 Albert Lasker medical research prize. The Lasker Award is most coveted and is often a prelude to a Nobel prize. Dr. Kandel received the award for his imaginative and innovative research on the manner in which the neural systems of lower organisms are changed during learning and retention of simple responses. Dr. Kandel is one of five researchers in the Learning and Memory Special Focus Program of the Office of Naval Research.

(Donald P. Woodward, ONR)

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